SUBSTANCE USE DISORDERS AND THE BEHAVIORAL EFFECTS OF NOVEL AND EXISTING COMPOUNDS

Sarah Uribe
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SUBSTANCE USE DISORDERS AND THE BEHAVIORAL EFFECTS OF NOVEL AND EXISTING COMPOUNDS

by

Sarah Uribe

A Thesis

Submitted to the
Department of Chemistry and Biochemistry
College of Science and Mathematics
In partial fulfillment of the requirement
For the degree of
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at
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Dedication

I dedicate this manuscript to my parents, Juan Uribe and Maria Gomez. My parents came to the United States 30 years ago from Colombia as two young adults searching for better opportunities. It is a privilege to be the first in my family to pursue a Master's Degree, and I want to express my gratitude for having the opportunity to do so. Thank you both for giving me the strength to reach for the stars and instilling in me to never give up, no matter how big or small a goal may be. If it were not for their support, love, and guidance, I would not be where I am today. I love you both endlessly; thank you for always believing in me.
Acknowledgments

I extend my gratitude to my mentor since my undergraduate studies, Dr. Thomas Keck, Ph.D. Thank you for your unconditional support and encouragement during my journey at Rowan University. Dr. Keck strives to make his students better scientists every day. He has taught me things I will continue to use in my future career and academic endeavors. I also want to thank Dr. Bradford Fischer, Ph.D., for his constant support and help during my research career. To Dr. Amanda Fakira, Ph.D., and Dr. Lark Perez, Ph.D., thank you for being a part of my thesis defense and educational journey. I also want to thank my fellow lab partners; I could not have done it without you all. Working with them provided me with countless memories and life lessons that I will be able to hold on to for the rest of my life. Finally, to my parents, family, and friends, every one of you has made an impact in pursuing my education; I am eternally grateful.
Abstract
Sarah Uribe
SUBSTANCE USE DISORDERS AND THE BEHAVIORAL EFFECTS OF NOVEL AND EXISTING COMPOUNDS
2021-2022
Thomas M. Keck, Ph.D.
Master of Science in Pharmaceutical Sciences

These investigations aimed to study the effects of experimental compounds for use in treating and preventing substance use disorders, particularly opioid use disorder (OUD) and alcohol use disorder (AUD). Identifying and testing potential medications for these two diseases is critical since current FDA-approved medications are only modestly effective, and addiction continues to spread. We previously demonstrated that co-administration of morphine and MP-III-024 produced synergistic effects in hot plate and von Frey assays. This study evaluated whether MP-III-024 also produced synergistic effects in behavioral tests sensitive to the side effects of morphine. Our findings suggest that co-administration of morphine and MP-III-024 at a 1.0:0.94 ratio produced sub-additive effects in morphine-induced hyperlocomotion, behavioral disruption measure in food-maintained operant responding, and conditioned place preference. Additionally, co-administration of MP-III-024 does not affect the development of analgesic tolerance caused by chronic morphine exposure. The second part of this study investigated whether cariprazine, a D3-preferring dopamine D3R/D2R receptor partial agonist, alters alcohol self-administration in mice. Our results show a sex difference in ethanol consumption, which made evaluation of cariprazine effects difficult to detect. Further studies would be necessary to reliably conclude whether cariprazine could effectively treat AUD.
# Table of Contents

Abstract ........................................................................................................................................... v  

List of Figures ................................................................................................................................. xi  

List of Tables .................................................................................................................................. xiii 

Chapter 1: Introduction to Substance Use Disorders ................................................................. 1  
  Substance Use Disorder .............................................................................................................. 1  
  History of Opioids ...................................................................................................................... 1  
  What are Opioids? ....................................................................................................................... 4  
  Intracellular Activity of Opioid Receptors ................................................................................. 5  
  Pharmacological Interactions at Opioid Receptors ................................................................... 6  
  How Opioids Produce Analgesia ............................................................................................... 7  
  Use of Opioid Drugs .................................................................................................................... 9  
    Analgesics ............................................................................................................................... 9  
    Opioids for Gastrointestinal Tract .......................................................................................... 9  
    Opioids for Chronic Cough ...................................................................................................... 10  
    Opioid Clinical Use as Anesthetic Agents ............................................................................ 10  
  Adverse Effects of Opioids .......................................................................................................... 11  
    Opioid-Induced Respiratory Depression ............................................................................... 11  
    Opioid-Induced Constipation ................................................................................................. 12  
    Opioid-Induced Nausea & Vomiting ....................................................................................... 12  
    Opioid-Induced Sedation ......................................................................................................... 13  
    Opioid-Induced Hyperalgesia ................................................................................................. 13  
    Opioid-Induced Hormonal Changes ....................................................................................... 14  
    Opioid-Induced Cardiac Effects .............................................................................................. 14  

vi
# Table of Contents (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opioid-Induced Sleep Disturbances</td>
<td>14</td>
</tr>
<tr>
<td>Tolerance</td>
<td>15</td>
</tr>
<tr>
<td>Dependence &amp; Withdrawal</td>
<td>15</td>
</tr>
<tr>
<td>OUDs Etiology</td>
<td>16</td>
</tr>
<tr>
<td>Diagnosis of Opioid Use Disorder (OUD)</td>
<td>16</td>
</tr>
<tr>
<td>Opioid Epidemic Waves</td>
<td>18</td>
</tr>
<tr>
<td>Pharmacotherapies</td>
<td>19</td>
</tr>
<tr>
<td>Opioid Addiction Treatment</td>
<td>19</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>20</td>
</tr>
<tr>
<td>Methadone</td>
<td>21</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>22</td>
</tr>
<tr>
<td>Behavioral Therapies</td>
<td>23</td>
</tr>
<tr>
<td>Cognitive Behavioral Therapy (CBT)</td>
<td>23</td>
</tr>
<tr>
<td>Contingency Management</td>
<td>23</td>
</tr>
<tr>
<td>Community Reinforcement Approach (CRA)</td>
<td>23</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>24</td>
</tr>
<tr>
<td>New “Ideal Analgesic” Approach</td>
<td>24</td>
</tr>
<tr>
<td>Fixed-Ratio Combinations</td>
<td>25</td>
</tr>
<tr>
<td>The Benefits of Combination Therapy</td>
<td>25</td>
</tr>
<tr>
<td>Research Goal</td>
<td>26</td>
</tr>
<tr>
<td>Alcohol</td>
<td>28</td>
</tr>
<tr>
<td>The History of Alcohol</td>
<td>28</td>
</tr>
</tbody>
</table>
Table of Contents (Continued)

Alcohol Content of Beverages ................................................................. 30
Pharmacokinetics of Alcohol (Ethanol) .................................................. 31
Neurotransmitters & Alcohol ................................................................. 32
Alcohol Use Disorder (AUD) ................................................................. 33
  DSM-5 Diagnosis of AUD ................................................................. 33
Pharmacotherapies for AUD ................................................................. 34
  Disulfiram ......................................................................................... 35
  Naltrexone ......................................................................................... 36
  Acamprosate ......................................................................................... 36
Research Goal ......................................................................................... 38

Chapter 2: Assessing the Possible Synergistic Effects of Morphine and MP-III-024 Co-
Administration on Food Self-Administration, Open Field Behavior, Conditioned Place
Preference, and Analgesic Tolerance ....................................................... 40
  Introduction ......................................................................................... 40
  Interactive Effects Morphine and MP-III-024 in Preclinical Models of Pain ........ 42
  Materials and Methods ........................................................................ 49
  Drugs .................................................................................................... 49
  Animals ................................................................................................. 51
  Behavioral Assays ................................................................................ 52
    Operant Conditioning ......................................................................... 52
    Open Field ........................................................................................ 53
    Conditioned Place Preference .......................................................... 55
    Hot Plate Assay ................................................................................ 56
Table of Contents (Continued)

Von Frey Assay .................................................................................................................. 58
Experimental Procedure ................................................................................................. 60
Food Self-Administration Procedure ........................................................................... 60
Open Field Procedure ..................................................................................................... 62
Conditioned Place Preference Procedure .................................................................... 65
Morphine Tolerance Assay ............................................................................................. 67
Data & Results .................................................................................................................. 71
Food Self-Administration Results .................................................................................. 71
Open Field Locomotor Results ...................................................................................... 75
Conditioned Place Preference Results .......................................................................... 79
Hot Plate Assay Results ................................................................................................. 81
Discussion ....................................................................................................................... 83

Chapter 3: The Effects of the Dopamine D3 Receptor Antagonist Cariprazine on Alcohol
and Palatable Food Self-Administration ....................................................................... 87

Introduction ..................................................................................................................... 87
Materials & Methods ...................................................................................................... 89
Drug ................................................................................................................................. 89
Drug Dosing .................................................................................................................... 90
Animals ............................................................................................................................. 92
Equipment ....................................................................................................................... 92
Operant Chambers .......................................................................................................... 92
Experimental Procedure ............................................................................................... 93
Food Self-Administration Procedure .......................................................................... 93
Table of Contents (Continued)

- Ethanol Self-Administration Procedure .................................................. 93
- Data & Results .............................................................................................. 94
- Food Self-Administration Results ................................................................. 94
- Ethanol Self-Administration Results ............................................................. 96
- Discussion ..................................................................................................... 98
- References .................................................................................................... 101
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1. Intracellular Changes After the Binding of an Opioid Agonist to an Inhibitory G-Protein Coupled Receptor</td>
<td>6</td>
</tr>
<tr>
<td>Figure 2. How Opioids Inhibit Transmission of Pain Signals</td>
<td>8</td>
</tr>
<tr>
<td>Figure 3. Structure of Buprenorphine</td>
<td>20</td>
</tr>
<tr>
<td>Figure 4. Structure of Methadone</td>
<td>21</td>
</tr>
<tr>
<td>Figure 5. Structure of Naltrexone</td>
<td>22</td>
</tr>
<tr>
<td>Figure 6. Variations of a Standard Alcoholic Drink</td>
<td>31</td>
</tr>
<tr>
<td>Figure 7. Structure of Disulfiram</td>
<td>36</td>
</tr>
<tr>
<td>Figure 8. Structure of Acamprosate</td>
<td>37</td>
</tr>
<tr>
<td>Figure 9. The Co-Administration of Morphine and MP-III-024 Attenuated Inflammatory Pain</td>
<td>45</td>
</tr>
<tr>
<td>Figure 10. Isobolographic von Frey Analysis</td>
<td>46</td>
</tr>
<tr>
<td>Figure 11. The Co-Administration of Morphine and MP-III-024 Attenuated Thermogenic Pain</td>
<td>47</td>
</tr>
<tr>
<td>Figure 12. Isobolographic Hot Plate Analysis</td>
<td>48</td>
</tr>
<tr>
<td>Figure 13. Structure of Morphine</td>
<td>50</td>
</tr>
<tr>
<td>Figure 14. Structure of MP-III-024</td>
<td>51</td>
</tr>
<tr>
<td>Figure 15 Self-Administration Chamber</td>
<td>53</td>
</tr>
<tr>
<td>Figure 16. Open Field Chambers</td>
<td>54</td>
</tr>
<tr>
<td>Figure 17. Conditioned Place Preference Chamber</td>
<td>56</td>
</tr>
<tr>
<td>Figure 18. Hot Plate Apparatus</td>
<td>58</td>
</tr>
<tr>
<td>Figure 19. Von Frey Apparatus</td>
<td>59</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Figure 20.</td>
<td>Tolerance Test Experimental Design</td>
</tr>
<tr>
<td>Figure 21.</td>
<td>Food Self-Administration Rewards</td>
</tr>
<tr>
<td>Figure 22.</td>
<td>Food Self-Administration Response Rates</td>
</tr>
<tr>
<td>Figure 23.</td>
<td>Effects of MP-III-024 on Food-Maintained Operant Responding</td>
</tr>
<tr>
<td>Figure 24.</td>
<td>Open Field Locomotor Results</td>
</tr>
<tr>
<td>Figure 25.</td>
<td>Comparison of Vehicle and MP-III-024 in Locomotor Activity</td>
</tr>
<tr>
<td>Figure 26.</td>
<td>Comparison of Morphine and the Different Drug Mixtures Tested</td>
</tr>
<tr>
<td>Figure 27.</td>
<td>Conditioned Place Preference Results</td>
</tr>
<tr>
<td>Figure 28.</td>
<td>Development of Morphine Tolerance as Measured by the Hot Plate Assay</td>
</tr>
<tr>
<td>Figure 29.</td>
<td>Structure of Cariprazine</td>
</tr>
<tr>
<td>Figure 30.</td>
<td>Food Self-Administration Cariprazine Rewards</td>
</tr>
<tr>
<td>Figure 31.</td>
<td>Food-Self Administration Cariprazine Response Rates</td>
</tr>
<tr>
<td>Figure 32.</td>
<td>Ethanol Self-Administration Cariprazine Rewards</td>
</tr>
<tr>
<td>Figure 33.</td>
<td>Ethanol Self-Administration Cariprazine Response Rates</td>
</tr>
</tbody>
</table>
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1. Morphine and MP-III-024 Drug Dosing: Latin Square Design</td>
<td>61</td>
</tr>
<tr>
<td>Table 2. Food Self-Administration Drug Dosing Ratios</td>
<td>62</td>
</tr>
<tr>
<td>Table 3. Open Field Test Drug Doses</td>
<td>64</td>
</tr>
<tr>
<td>Table 4. Open Field Drug Dosing Ratios</td>
<td>65</td>
</tr>
<tr>
<td>Table 5. Conditioned Place Preference Drug Doses &amp; Groups</td>
<td>67</td>
</tr>
<tr>
<td>Table 6. Tolerance Assay Experimental Drug Design</td>
<td>70</td>
</tr>
<tr>
<td>Table 7. Cariprazine Drug Dosing: Latin Square Design</td>
<td>90</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction to Substance Use Disorders

Substance Use Disorder

A substance use disorder (SUD) is a chronic illness characterized by poor control over one's use of substances, including alcoholic beverages, illegal drugs, and prescribed medications. According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), SUDs include symptoms of tolerance, dependence, withdrawal, and an uncontrolled desire to increase intake. With more individuals suffering the effects of substance abuse, there is a dire need for scientists to develop medications with fewer adverse effects on humanity aimed to combat these disorders. As a result of the opioid epidemic that began in the early 1990s, SUDs have become increasingly prevalent in the United States. The opioid epidemic has raised awareness of the need for new molecular and behavioral pharmacology tools. The Keck Laboratory at Rowan University aims to discover and test new potential medications for drug addiction, schizophrenia, bipolar disorder, pain, and other medical conditions. This specific research project aims to gather scientific knowledge to improve treatment for SUDs such as OUD: the use of opioids in a manner that leads to impairment or distress, and AUD, which is a tendency to drink excessively and to be preoccupied with alcohol.

History of Opioids

The use of opium, known as the "natural" source of opiates, dates back to ancient civilizations, which faces the challenge of balancing the analgesic and euphoric effects of
opiates, resulting in its misuse and abuse. Opioids belong to a large class of medications derived naturally from the opium poppy plant, *papaver somniferum*. Among the earliest references to opium use are from lower Mesopotamia in Southwest Asia, which dates back to 3,400 B.C. The Sumerians called opium the “joy plant” shortly after, the Sumerians brought opium to the Assyrians and Egyptians.\[3\]

In the 15th and 16th centuries, opium made its way to Europe, where Europeans consumed opium as a therapeutic agent for illnesses and psychological disorders. In the early 1800s, German pharmacist Friedrich Seturner discovered modern-day opioid pharmacology. Originally named after the Greek god of dreams, Morpheus, the German pharmacist isolated and extracted the alkaloid from poppy plants, which led to the creation of the standard drug known as morphine.\[3\] Shortly after, morphine became a prescribed drug in the U.S. by doctors for treating pain, anxiety, and respiratory issues. In 1853, the hypodermic needle was introduced, and morphine became a widely used medication for procedures for neuralgia. Furthermore, when the Civil War began in the 1860s, injured soldiers were administered morphine to treat pain. As a result, they started to develop opioid dependencies, precipitating the public health crisis known as “Soldier's disease”.\[4,5\]

In 1874 the British chemist Charles Romley Alder Wright synthesized heroin from morphine. He introduced it as a cough suppressant to the Bayer Company of Germany in 1898. Surprisingly, it was offered and marketed as a “non-addictive” substitute for morphine and had promising clinical results to the extent that it was labeled the wonder drug. However, after being released on the market, the medical personnel began noticing that patients prescribed heroin were becoming very dependent and
building a tolerance to the drug. As a result, larger doses became necessary to produce a therapeutic effect after administration. After they established its highly addictive properties, there was a total ban on heroin production and sale, which resulted in a significant decrease in consumption after the 1930s. [6] Moreover, in 1995 the American Pain Society reviewed and expanded guidelines for the treatment of acute and cancer pain in order to improve the quality of pain management. Near the same time, Purdue Pharma released a new formulation of oxycodone known as OxyContin™. Between 1997 and the early 2000s, OxyContin™ prescriptions in the United States increased from 670,000 to 6.2 million annually. A decade later, Purdue Pharma pleaded guilty to federal charges of misbranding the drug, misleading physicians and the medical community about the adverse effects and failing to disclose the risks associated with these drugs. [4,5] The misuse and abuse of opioids, particularly oxycodone, led to the first wave of the opioid epidemic in the United States, causing increasing deaths each day due to overprescribing opioids to patients.

Increasingly, the opioid epidemic is now driven by synthetic opioids that are highly potent. A notable synthetic opioid is fentanyl, which is more than 100 times more potent than morphine. Given the gravity of the public health issue, various laws were put in place to help the crisis, one of which denied any practitioner the right to prescribe or dispense any opioid for acute pain to an opioid-naive patient for more than a seven-day supply. Consequently, the United States suffered and continues to suffer, noting that 2020 was the deadliest year yet in the North American Opioid epidemic. [7]
What are Opioids?

Analgesics such as opioids can be divided into naturally occurring opiates and synthetic derivatives. Opioids are highly potent and effective analgesics but come with a high degree of dependence and abuse. Opioids activate three classical opioid receptors: the µ-opioid receptor (MOR), κ-opioid receptor (KOR), and δ-opioid receptor (DOR). These receptors belong to the large superfamily of seven transmembrane-spanning (7TM) G protein-coupled receptors (GPCRs). GPCRs are known to mediate the actions of neurotransmitters and hormones. In 1994, a new G protein-coupled endogenous opioid-like receptor was discovered, known as the nociceptin (NOP) receptor, introducing the fourth opioid receptor; nociceptin/orphanin FQ (N/QFQ). These receptors can be found in the central nervous system (CNS) and peripheral nervous system (PNS).

Furthermore, opioids can be divided into two types: endogenous opioids and exogenous. Endogenous opioid peptides are short amino acid chains that function as hormones and neuromodulators. Examples of endogenous opioid peptides include enkephalins, endorphins, dynorphins, and nociceptin/orphanin. Examples of exogenous opioids include morphine, heroin, and fentanyl, which bind to the same receptors as endogenous opioids. These drugs are either naturally occurring, semi-synthetic or fully synthetic opioids. Each of the four opioid receptors produces various effects, from analgesia to respiratory depression, nausea, and vomiting, limiting their therapeutic utility.
Intracellular Activity of Opioid Receptors

As Gα\(_{i/o}\)-coupled GPCRs, MOR, DOR, and KOR, display similar cellular responses following receptor activation. As shown in Figure 1, binding of an opioid agonist to the transmembrane region of the GPCR causes the G protein’s α subunit to exchange its bound guanosine diphosphate (GDP) molecule with intracellular guanosine triphosphate (GTP). Following this, the αGTP dissociates away from the βγ complex, allowing the G protein subunits to interact with other target proteins. Gα\(_{i/o}\) negatively regulates the enzyme adenylyl cyclase, causing a reduction in intracellular cyclic adenosine monophosphate (cAMP) levels. cAMP regulates Protein Kinase A (PKA), which is responsible for the phosphorylation of proteins, ion channels, and enzymes through activation or inhibition; reducing cAMP causes a decreased activity in neurotransmitter release. The βγ complex activates GIRK channels, increasing potassium conductance, leading to hyperpolarization in neuronal cells, and a reduction in neuronal firing.\(^{[10,11]}\)
Figure 1 [11]

_Intracellular Changes After the Binding of an Opioid Agonist to an Inhibitory G-Protein Coupled Receptor_

_Note_. Figure adapted from Hasan et al. (2012).

**Pharmacological Interactions at Opioid Receptors**

In addition to their different classification groups, opioids have different signaling properties and can produce different effects.

1. **Full Agonists:** are classified as compounds that evoke a maximal defined response at the opioid receptors. Examples of full agonists include morphine, oxycodone, and methadone. [13,14]
2. **Partial agonists:** are classified as compounds that activate receptors with a partial response at the opioid receptors. Examples of partial agonists include buprenorphine, tramadol, and butorphanol. [13,14]

3. **Antagonists:** are classified as compounds that bind to receptors to block or reverse the effects of opioids. Examples of Antagonists include naltrexone and naloxone. [13,14]

**How Opioids Produce Analgesia**

Signal transmissions can occur through the ascending and descending signaling pathway. At the sight of injury, the pain signal coming from the injury will travel up to the brain through the ascending signaling pathway, which allows us to perceive pain. The signal will travel through the dorsal horn to the thalamus which then relays this signal to different areas in the brain to coordinate an appropriate response. The descending pathway is responsible for controlling the pain signal. Once the brain coordinates a response, these pain signals are then sent back to the spinal cord. The two important areas in the descending pathway include periaqueductal gray (PAG) and the nucleus raphe magnus (NRM).

The activation of opioids triggers neuronal changes, leading to pleasurable effects, an inability to feel pain, and analgesia. Three regions in the brain contain a particularly high concentration of opioid receptors: the periaqueductal gray (PAG), the locus coeruleus, and the rostral ventral medulla. In **Figure 2** the illustration shows how MOR agonists indirectly stimulate the descending inhibitory pathways; particularly the PAG and nucleus reticularis paragigantocellularis (NRPG). This activation causes inhibitory
neurons in the pathway, in addition to an increase in neuronal traffic through the nucleus raphe magnus (NRM). The process of analgesia results in reduced nociceptive transmission from the periphery to the thalamus. Thus, these cellular occurrences produce an analgesic effect after administering a MOR agonist. \[11\]

**Figure 2** \[11\]

*How Opioids Inhibit Transmission of Pain Signals*

Note. MOR agonists produce analgesia by indirectly increasing neuronal traffic through the descending pathway at the NRPG and PAG or directly inhibiting nociceptive afferents in the periphery. MOR agonists act at the NRM to indirectly inhibit spinal pain transmission and reduce spinal nociception. PAG = periaqueductal gray; NRPG = nucleus reticularis paragigantocellularis; NRM = nucleus raphe magnus; LC = locus coeruleus. Figure adapted from Hasan *et al.* (2012)
Use of Opioid Drugs

Analgesics

Opioids have been a part of the standard care treatment for acute and chronic pain for many years. Chronic pain can be categorized as either nociceptive or neuropathic pain caused by the central or peripheral nervous system. Nociceptive pain is associated with external stimuli causing damage to body tissue. Neuropathic pain is developed in the nervous system caused by an injury or disease of the somatosensory system. [17] Recently the center for disease control and prevention (CDC) released an updated 2022 clinical practice guideline for prescribing opioids for pain. These new guidelines aim to implement safe and effective measures for pain management. Studies found that nonopioid therapies were just as effective as prescribing opioids for many types of acute pain, including lower back pain, neck pain, headaches, common musculoskeletal conditions, and minor surgeries. Clinicians must implement nonopioid therapies, such as topical or oral anti-inflammatory medications and exercise, as appropriate for each patient's condition. Nonetheless, they are aware that there is still a need for opioid therapy for moderate-to-severe acute pain when nonopioid therapies are ineffective. [18]

Opioids for Gastrointestinal Tract

Opioid agents are also used to treat the gastrointestinal tract, particularly by treating severe diarrhea and controlling high-output ostomies. The two most commonly used medications for diarrhea are loperamide and diphenoxylate. The third FDA-approved medication is difenoxin, a metabolite of diphenoxylate. Loperamide is available as an over-the-counter drug without prescription; the other two are prescription drugs.
classified as controlled substances (Schedule V and Schedule IV) due to having a higher potential for misuse and abuse.\textsuperscript{[19-21]} Opioids are known to inhibit gastric emptying and peristalsis in the GI tract which leads to constipation. However, endogenous opioids are known to play a critical role in GI signaling which cause changes in motility, secretion and the transport of fluids and electrolytes. After the binding of these opioids, G-protein receptor kinases, phosphorylation, binding of β-arrestin proteins, endocytosis through inactivation of ADP-ribosylation factor and recycling at different rates occur allowing these medications to aid the gastrointestinal tract.\textsuperscript{[101]}

\textit{Opioids for Chronic Cough}

Many common inflammatory airway diseases can be associated with chronic cough, such as asthma, chronic obstructive pulmonary disease (COPD), post-viral infections, pulmonary fibrosis, and bronchiectasis.\textsuperscript{[22]} Inflammatory airway diseases can be treated with angiotensin-converting enzyme (ACE) inhibitors, nonsteroidal anti-inflammatory drugs (NSAIDs), and inhalant medications. Opioids have also been used as antitussives, known as cough suppressants, which suppress the μ and κ opioid receptor agonism. A popular cough suppressant is codeine, commonly taken with other drugs such as acetaminophen; they work together to treat moderate pain and reduce coughing.\textsuperscript{[23-26]}

\textit{Opioid Clinical Use as Anesthetic Agents}

Anesthesiologists still use various opioids to achieve different anesthetic plans for a therapeutic or surgical procedure. Opioids are given as preanesthetic, postoperative analgesia, and intraoperative for general anesthesia. The receptors in the CNS cause desirable effects, including analgesia, sleepiness, and suppression of reflex responses to
noxious stimuli. The suppression of certain reflexes may also reflect the patient's tolerance for airway manipulation, endotracheal intubation, and mechanical ventilation.\(^{[26]}\)

**Adverse Effects of Opioids**

**Opioid-Induced Respiratory Depression**

Opioids effectively treat pain. However, opioids have several side effects, particularly after prolonged use, resulting in many adverse consequences. One of the most life-threatening side effects of opioid use is respiratory depression. Opioid-induced respiratory depression (OIRD) can lead to death, particularly when opioids are abused and misused with other sedatives, such as sleep medication or alcohol, or when combined with other illicit substances.\(^{[27]}\) The human population depends on the cardiorespiratory control system to breathe and survive, needing oxygen and carbon dioxide removal through our lungs. The two systems that control ventilation are 1) the chemical or metabolic control of breathing and 2) the behavioral control system. The chemical system depends on the chemical composition of arterial blood and cerebrospinal fluid, such as pH, carbon dioxide (CO\(_2\)) partial pressure (pCO\(_\text{2}\)), and oxygen partial pressure (pO\(_\text{2}\)). The behavioral control system is active during wakefulness and rapid-eye-movement sleep, allowing breathing to be specific circumstances such as eating, speaking, anxiety, and pain.\(^{[27,28]}\) Opioids like morphine activate MORs expressed on respiratory neurons in the medulla and pons, which lowers the body’s rate at which the brain can detect changes in CO\(_2\) levels. This suppression is the leading cause of many of the deaths associated with OIRD.\(^{[9,28]}\)
**Opioid-Induced Constipation**

Another common adverse effect is opioid-induced constipation (OIC), which many patients experience when they are on opioid therapy for chronic pain. Since the alimentary canal is equipped with the largest collection of neurons outside the brain and the enteric nervous system, many drugs have an effect on the gut. Upon release from enteric neurons, opioid peptides modify the gastrointestinal tract (GI) by interacting with opioid receptors that are located on enteric circuits that control secretion and motility. The GI tract is interrupted by opioid receptor agonists in excitatory and inhibitory neural inputs to the GI muscle. When the excitatory pathways are suppressed, the neurotransmitter acetylcholine blocks peristaltic contractions. In contrast, blocking the inhibitory neural inputs leads to a depression of nitric oxide release from inhibitory motor neurons, causing an increase in GI muscle activity, the elevation of resting muscle tone and non-propulsive motility patterns. \[19\] Thus, most patients suffering from this side effect complain of straining and emptying their rectum while defecating. \[9,30\]

**Opioid-Induced Nausea & Vomiting**

Opioid administration can commonly induce nausea and vomiting, leaving patients with worse symptoms than pain. The cause of nausea and vomiting involves CNS and PNS mechanisms. After administration, the chemoreceptor trigger zone (CTZ) inhibits gut motility and stimulates the vestibular apparatus. Researchers have found that the neurokinin-1 (NK-1) and serotonin receptors in the postrema area are involved in this opioid-induced emesis. Although opioids continue to be a commonly prescribed drug to
treat analgesia, these situations have led researchers to prescribe a serotonin receptor agonist and an NK-1 antagonist to help patients with the adverse effects of opioids. \cite{30,31}

**Opioid-Induced Sedation**

The pharmacology of opioids contributes to sedation and decreased cognition during opioid treatment. Opioid sedation occurs in 20-60% of patients receiving opioids, disrupting the quality of life in an individual. Although the mechanism is poorly understood, it is common to be given a psychostimulant to manage this side effect. Additionally, this side effect is dose-dependent, and patients acquire a tolerance to sedative effects. When opioid receptors in the central nervous system are activated, they inhibit the firing of neurons, which inhibits the arousal mechanism causing decreased wakefulness and slowed interpretation. \cite{32}

**Opioid-Induced Hyperalgesia**

As a result of opioid-induced hyperalgesia (OIH), nociceptive sensitization is acquired, causing a more acute response to certain painful stimuli. The mechanism of action of OIH is still being studied, and they believe that neuroplastic changes in the CNS and the PNS lead to this sensitization of pronociceptive pathways. Pain will still reside regardless of opioid doses being increased due to the opioid metabolite morphine 3-glucuronide (M3G) causing hyperalgesia. N-methyl-D-aspartate (NMDA) receptor agonism can also play a role in developing hyperalgesia; glycine mediates postsynaptic inhibition of spinal neurons. \cite{33,34}
**Opioid-Induced Hormonal Changes**

Opioids cause opioid endocrinopathy, suppressing the body’s hormonal system in both men and women. The reported long-term effects of treatment caused by the endocrine system are sexual dysfunction, amenorrhea, fatigue, low testosterone, and depression. Clinically, these side effects have been more relevant in males. [35]

**Opioid-Induced Cardiac Effects**

Patients have reported decreased cardiac function on opioid therapy. Morphine causes histamine release resulting in vasodilation and hypotension. Methadone and buprenorphine have been shown to have prolonged QTc, and more so with patients at risk for prolonged QTc. In the case of patients having pre-existing conditions, patients receiving these medications must be monitored. [36]

**Opioid-Induced Sleep Disturbances**

Although there have been many reported studies on individuals abusing opioids for chronic pain as sleeping aids, there have also been studies stating that it contributes to insomnia. Opioid therapy leads to an increase in the number of shifts in sleepwalking states and an overall decrease in total sleep time, sleep efficiency, delta sleep, and rapid eye movement sleep (REM). Numerous studies show that the neurotransmitters involved in sleep include acetylcholine, dopamine, histamine, gamma-aminobutyric acid (GABA), pituitary hormones, and the neurohormone melatonin, noradrenaline, and serotonin. Although the mechanism of action is poorly understood, opioids like morphine appear to reduce REM sleep. It has been hypothesized that GABAergic signaling through inhibiting acetylcholine release in the medial pontine reticular formation causes sleep disruptions in
the basal forebrain. Additionally, the opioid peptides and the peptide neurohormone vasopressin participate in the induction and maintenance of the sleep state; driven by the suprachiasmatic nuclei.

**Tolerance**

Chronic opioid use leads to tolerance, a condition where the body gets used to the medicine, and either needs more medicine or a substituted agent to achieve the medicine effect. It has also been observed that tolerance to the analgesic effects of opioids may be an uncommon primary cause of declining analgesic effects in clinical settings; patients are experiencing worsening of their pain from dose escalation. There are two types of tolerance: short-term or acute, a tolerance that develops within minutes to several hours, and long-term or permanent tolerance that develops over a prolonged period of time.

**Dependence & Withdrawal**

The area most affected when opioid withdrawal effects reside is in the locus coeruleus. When an individual experiences physical dependence, usual withdrawal effects can be caused by the termination of an opioid, rapid dose reduction, or taking an opioid antagonist. Methadone and buprenorphine are common long-term replacements for managing opioid withdrawal; however, they remain ineffective. The ineffective treatments have led individuals to go back to old habits.
OUDs Etiology

OUD is characterized as the inability to abstain from using opioids, resulting in clinically significant distress and impairment in daily life. The use of opioids around the world affects more than 16 million people and over 2.1 million in the United States, attributing to 120,000 deaths worldwide. The American Psychiatric Association (DSM-5) diagnoses OUD as seeking to obtain and take opioids despite social and professional consequences. OUDs occur in individuals from all populations, not excluding any educational or socioeconomic backgrounds. Many factors can contribute to developing addiction; it has been studied that being deficient in certain neurotransmitters, such as dopamine, can cause addictive behaviors, as they need to seek external endorphins. Studies have also suggested that patients with substance abuse disorders in their families have about a 50% chance of inheriting these disorders. Another significant influence is our environmental atmosphere; patients suffering from a variety of mental illnesses are more susceptible to suffering from opiate use. Sources have also shown that genetics can play a role in developing these addictions leading to OUD. [41]

Diagnosis of Opioid Use Disorder (OUD)

The DSM-diagnostic criteria for OUD states that in order to receive a diagnosis of an OUD at least two of the following should be observed within a 12-month period. [41]

A list of the eleven problems:

1. Taking large quantities of opioids over a longer extended period than intended
2. Ongoing opioid use & unsuccessful efforts to cut down and control opioid use
3. Cravings, having a strong urge to use opioids

4. Not fulfilling professional obligations at school or work

5. Spending excessive time trying to gain access to opioids or recover from taking them

6. Continued opioid use despite social and interpersonal consequences

7. Undesire for social or recreational activities due to opioid use

8. Recurrent opioid use in situations in which it is physically hazardous

9. Continued opioid use despite physical or psychological consequences caused by opioids

10. Tolerance; is the need for increased amounts of a drug or diminished effects with continued use of the same amount

11. Experiencing withdrawal or consuming other substances to relieve withdrawal symptoms

Additional screening is necessary when a patient is diagnosed with an OUD, such as a complete history of the patient's social and mental health. Patients may also experience a variety of opioid withdrawal symptoms, some of which include cravings, diarrhea, anxiety, and agitation. Opioid intoxication can include symptoms of confusion, miosis, hypersomnia, nausea, euphoria, and overall decreased pain perception.\(^{42}\)
**Opioid Epidemic Waves**

The opioid crisis began in the early 1990s due to increased misuse, abuse, and overdose of opioids. According to the Centers for Disease Control and Prevention (CDC), opioid deaths have occurred in three waves since the 1990s: prescription opioids, heroin, and synthetic opioids. Recent studies suggest that we have entered a fourth wave, stimulant/opioid epidemic, with mental illness comorbidities.\[43,98\] The first wave of the opioid epidemic started in the early 1990s when doctors began overprescribing opioids and giving patients misinformation stating that these drugs were not addictive and would cause no problems. Despite not wanting to abuse their medications, some people become physically dependent on them and cannot stop and reduce their usage. Later, a campaign was released treating pain as the “fifth vital sign,” highlighting the importance of improved care. Additional vital signs noted were blood pressure, pulse, respiratory rate, and temperature. This campaign allowed the pain to be viewed as subjective, where a higher increase of opioids was needed to reduce pain. As a result of the second wave in 2010, many doctors were getting in trouble for prescribing opioids, and it began to reduce their prescriptions; however, it was too late. Since many patients had developed an addiction, people turned to heroin to help control their withdrawal symptoms and give them a feeling of euphoria. Heroin is known for being even more addictive than opioids, as it continues to be laced with other drugs making this substance much more dangerous.\[43,44,98\]

The third wave started in 2013, when fentanyl, a synthetic opioid, became the popular drug of choice. This wave was one of the most dangerous as fentanyl is 50 to 100 times stronger than morphine, being extremely potent at lower doses. Unfortunately, due
to its street sale, it was mixed with other drugs to increase its effectiveness and profits, becoming much more dangerous. [43,44] This tactic increased overdose deaths, contributing to the opioid epidemic.

Due to a lack of stimulation intervention tools, the difficulty of addressing multi-substance abuse, and the impact of the COVID-19 epidemic, the current wave of the opioid epidemic in the United States is much more severe than previous waves. A National Survey on Drug Use and Health conducted in 2020 states that methamphetamine use increased in individuals 26 and older from 2016 to 2019. This survey also revealed that many individuals reported co-occurring mental illnesses while simultaneously consuming illicit drugs. Of those individuals, only 12.7% of them received services for both of their disorders. [98] The CDC mentions that since the COVID-19 pandemic, fentanyl overdoses have increased by 30% in a year. Nonetheless, the cases continue to increase due to reduced access to healthcare, recovery, and support services. [45]

**Pharmacotherapies**

**Opioid Addiction Treatment**

Opioid addiction has been treated with FDA-approved OUD medications and/or behavioral therapy. Unfortunately, regardless of the behavioral and pharmacotherapies available, the rate of opioid use after treatment continues to increase, making the need for new analgesics vital. A combination of psychosocial interventions with medications is known as medication-assisted treatment (MAT); studies show promising outcomes when treating patients with OUD; however, abuse and misuse continue to be problems. Currently, the three FDA-approved drugs for OUD treatment are buprenorphine,
methadone, and naltrexone. Available behavioral therapies include cognitive behavioral therapy, contingency management, and community reinforcement. [46]

**Buprenorphine**

As an opioid agonist, buprenorphine reduces the desire to use the problem drug by reducing cravings and attenuating opioid withdrawal symptoms. Clinically, buprenorphine acts like a typical MOR agonist; however, at higher doses, the buprenorphine’s MOR agonist effects reach a plateau, and its KOR antagonist effects begin. The effect of this drug decreases its abuse potential and risk of overdose, even at higher doses. Buprenorphine is taken either alone or in combination with the antagonist naloxone, a formulation designed to further limit abuse. [46]

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**Figure 3** [104]

*Structure of Buprenorphine*
Methadone

The opioid full agonist methadone is used to treat chronic pain and opioid addiction, reducing withdrawal symptoms and cravings. Increasing methadone doses lead to opioid tolerance, reducing reinforcement from other opioids, such as heroin, through cross-tolerance. Medications such as methadone have been used in opioid maintenance and detoxification for many years. This medication decreases withdrawal effects while causing the patient to feel normal. The combination of this medication and behavioral therapy is the most effective.\textsuperscript{[46]}

Figure 4\textsuperscript{[105]}

Structure of Methadone
**Naltrexone**

Naltrexone is a competitive opioid antagonist with a higher affinity for MOR receptors and a lower affinity for DOR and KOR receptors. Naltrexone is commonly used to prevent relapse but may not entirely stop drug cravings. The patient should not take opioids before taking naltrexone since the body can easily experience withdrawal symptoms from the drug. Naltrexone is considered the “ideal” drug for relapse prevention of OUD due to its favorable adverse effect profile and pharmacological properties. This therapy's drawback is that it prevents the opioids' rewarding effect, which results in behavior extinction. \[46\]

**Figure 5** \[106\]

*Structure of Naltrexone*
Behavioral Therapies

*Cognitive Behavioral Therapy (CBT)*

Cognitive behavioral therapy aims to prevent relapse and address other problems that can co-occur with drug abuse and stop drug abuse by identifying and correcting certain behaviors. Participants in this therapy learn adaptive strategies such as the pros and cons of continued drug use, self-monitor their drug cravings, and identify situations that may put them at risk. \[47\]

*Contingency Management*

Contingency management (CM) focuses on offering patients’ tangible benefits to reinforce desirable behaviors such as abstinence. In this therapy, the two physical rewards are a prize incentive and voucher-based reinforcement (VBR). The different incentives can be exchanged for food items, movie passes, or drug-free goods or services; as there is an increase in the number of received drug-free urine samples, the incentive also increases. In addition, research has found that VBR promotes abstinence from opioids and cocaine in patients undergoing methadone detoxification and behavioral therapies. \[47\]

*Community Reinforcement Approach (CRA)*

Community Reinforcement consists of an intensive 24-week outpatient therapy using recreational, familial, social, and vocational reinforcement and material incentives. This program aims to keep abstinence long enough to adopt new lifestyle skills and eliminate substance abuse. Patients need to submit urine samples twice each week to
receive an incentive. Studies have shown that urban and rural areas have found engagement in treatment and patients gaining periods of abstinence. [47]

Benzodiazepines

Benzodiazepines are a class of drugs that act upon benzodiazepine receptors in the central nervous system. They are prescribed for psychoactive disorders such as anxiety, insomnia, and acute status of epilepticus, and seizures. These drug types carry risks of increased sedation, cognitive impairment in addition to respiratory depression. [100] Benzodiazepines bind between α/γ GABA sites, that potentiating GABA’s agonist effects, making them a positive allosteric modulators (PAMs). The binding of GABA at the GABA_A receptor’s agonist site and the benzodiazepine-receptor agonist at the PAM site increases the opening of the chloride channel, hyperpolarizing the neuron and blocking the action potential. [15,16]

New “Ideal Analgesic” Approach

Analgesics are a class of medications used to manage and treat pain. Several medications include acetaminophen, nonsteroidal anti-inflammatory drugs, antidepressant, antiepileptics, anesthetics, and opioids. The abuse potential of opioids has made it necessary to develop new treatment regimens and strategies for pain management. [48] Through the years, combination therapy has been a beneficial approach for treating many diseases. Combined drug therapy allows for an increased analgesic effect at lower doses of each constituent, reducing adverse effects overall and promoting synergism against a particular target. [49] Scientists are still searching for the “ideal analgesic,” a full agonist that provides optimal and maximal analgesia for many pain
states. The new strategies include altering existing analgesics or combining existing analgesic compounds with other compounds to improve the efficacy and reduce adverse effects that come with drugs like opioids. Drug combinations can be classified mechanistically as undesirable (sub-additive), desirable (additive), or highly desirable (synergistic). Combination therapy is a well-known research avenue that may provide pain medication with an improved analgesic profile. Combination studies with reduced side effects, including abuse potential, have been relatively unexplored compared to other research areas.

**Fixed-Ratio Drug Combinations**

In clinical settings, it is becoming increasingly popular to use analgesic combinations, using two different methods, fixed-dose ratios or *ad hoc* dose-ratios, to treat different types of pain, particularly when managing “mixed” pain disorders. The *ad hoc* method provides customizable therapy; however, the difficulty with this treatment method is maintaining dose ratios within the optimal range. The second method, fixed-ratio dose combinations, has been shown to facilitate producing more reproducible and standardized clinical results. The Keck animal behavioral laboratory also uses fixed-ratio dose combinations in its studies.

**The Benefits of Combination Therapy**

1. Find a combination to enhance or optimize analgesic efficacy (synergy)
2. Find combinations to diminish or minimize adverse effects
3. Find combinations to diminish opioid effects which are not beneficial
4. Find combinations to help reduce opioid tolerance/hyperalgesia

5. Find combinations to help with dependency and addiction

**Research Goal**

The initial research question is whether we can find new candidate medications that can be co-administered with opioid analgesics to enhance analgesia selectively, thus reducing overall exposure and the risks of opioid-induced side effects. Opioids are a broad class of drugs critical for treating acute and chronic pain, but their addictiveness limits their medical use as an analgesic. Due to the misuse and abuse of prescription and illicit drugs, there is an urgent need to develop novel analgesic treatment strategies. It is well established that MOR activation in the central and peripheral nociceptive pathways primarily mediates opioid analgesia, but also opioid side effects, including tolerance, constipation, respiratory depression, and abuse liability.\(^{[51]}\)

Antinociception can be managed by selectively enhancing \(\gamma\)-Aminobutyric acid (GABA) signaling at ionotropic GABA type A (GABA\(_A\)) receptors. From the six known alpha subunits found in the central nervous system, GABA\(_A\) receptors containing \(\alpha2\) and \(\alpha3\) subunits (\(\alpha2/\alpha3\)GABA\(_A\)) are co-expressed with MORs in the dorsal horn spinal pathways, which is vital for nociceptive transmission. The Keck behavioral laboratory and other collaborators have investigated the antihyperalgesic effects of \(\alpha2/\alpha3\)GABA\(_A\) Positive Allosteric Modulators (PAMs). Research has found that \(\alpha2/\alpha3\)GABA\(_A\) can be selectively targeted with novel imidazobenzodiazepine PAMs, such as MP-III-024, our drug of interest. MP-III-024 alone can produce some antinociceptive effects and produces a limited amount of behavioral disruption or sedation.\(^{[51, 52]}\)
Rahman et al. tested the MOR morphine alone or in combination with MP-III-024, reporting that co-administration produced synergistic effects in animal pain models. Following the findings of this study, we asked this follow-up question: does MP-III-024 selectively enhance morphine-mediated analgesia, or is it universally synergistic with morphine, producing a synergistic side effect profile? We hypothesized that since morphine and MP-III-024 have synergistic antinociceptive effects, MP-III-024 will enhance all of morphine’s side effects. To test our hypothesis, we tested varying doses of morphine alone (3.2, 10, and 32 mg/kg), MP-III-024 alone (3.2, 10, and 32 mg/kg), and different combination ratios (1.0:0.31, 1.0:0.94, and 1.0:2.8 morphine:MP-III-024) in models sensitive to various opioid side effects. Our studies followed the same dosing regimen that Rahman et al. used for his studies. The first test determined whether MP-III-024 potentiated morphine-induced disruptions of operant food self-administration. The next test evaluated whether MP-III-024 potentiated morphine-induced hyperlocomotion. To investigate abuse liability, we tested whether MP-III-024 potentiated morphine-induced conditioned place preference (CPP). Finally, we tested whether MP-III-024 potentiated the development of tolerance in the hot plate assay following chronic morphine.

Overall, these studies aim to clarify whether this new candidate drug type may be combined with opioids to improve analgesia while reducing side effects. If successful, we believe this dual-pharmacology strategy could reduce the risks of clinical opioid treatment and reduce the long-term danger of developing opioid dependence.
Alcohol

Alcohol is one of the most widely used recreational drugs, a chemical substance found in drinks such as beer, wine, and liquors. The type of alcohol used to make alcoholic beverages is known as ethyl alcohol or ethanol. Alcohol is made by fermentation, a biochemical process that converts sugar into cellular energy, ethanol, and carbon dioxide, among other metabolic products. Alcoholic fermentation is considered an anaerobic process since yeast performs this conversion without oxygen.

The History of Alcohol

Many cultures and civilizations have been influenced by alcohol, including the Sumerians, Egyptians, Greeks, Romans, Chinese, and the British. Alcoholic beverages have been fermented from grains and fruits for thousands of years, dating back to the earliest evidence of alcohol discovery before 3000 B.C. Evidence reports that Sumerians discovered over twenty beer recipes on clay tablets with specific rules and regulations on the consumption of alcohol. Sumerians also offered alcohol in sacrificial and religious environments to their gods.

The ancient Egyptians were known for their daily consumption of bread and beer, where beer, which consisted of barley, wheat, and yeasty dough, was regarded as the drink of the gods. Most Egyptians drank beer for its assumed nutritional benefits; moreover, beer was used as a remedy during their time. Following the discovery of beer, ancient Greece was one of the first known centers of wine production. As early as 2000 B.C.E., vineyards were established in Greece, where alcohol played an important role in religious ceremonies. As with other countries' influences and rituals, it was used
medicinally for lethargy, diarrhea, childbirth pains, and wound cleansing. The importance of wine in the Greek community led to the creation of a god, Dionysus. Dionysus is known as the god of fertility, ritual madness, and joy, representing living and dead. During these ancient Greek times, they would gather for what was known as a *symposium*, where elite men would socialize and drink, sharing different conversations and debates. Plato's Symposium, Homer's Iliad, and Homer's Odyssey emphasize the ancient relationship between drinking and celebration that remains with us to this day. Aside from adopting Greek wine culture, the Romans also used wine to exchange slave labor for goods. Over time, Romanians changed their attitudes toward drinking, which became a normal part of life for military personnel.\[55\]

Among the first countries to distill spirits with yeast-fermented bases was China. The natural fermentation of fruits and flowers produces alcohol, both of which are natural sources of the substance. According to Chinese tradition, alcohol has medicinal properties that relieve headaches and colds, strengthening the immune system.

The introduction of alcohol to Britain occurred during the 16th and 17th centuries. Additionally, between 1525 and 1550, distilled spirits became popular. Shortly after, in 1606, “drunkenness” was introduced as a crime, and the English Parliament passed The Act to Repress the Odious and Loathsome Sin of Drunkenness. In 1643, Britain began to tax their alcohol, which increased the growth of the moonshine trade, a high-proof liquor sold illegally. A popular beverage today, gin, was discovered in Holland in 1650. Following that, different whiskeys were discovered in Scotland and Ireland in the 1700s.\[55\]
Consuming alcoholic beverages came to North America before Christopher Columbus; however, with the arrival of the Europeans, their alcohol influence was adopted into American culture. During the 1800s, the average individual consumed fewer than two bottles of 80-proof liquor per week. Alcohol consumption continued to grow in America, one of the reasons being that it was healthier to consume alcohol than water. The abuse of alcohol led to a Prohibition enacted in the U.S., making alcohol illegal in 1919, but it continued in the trade market. Prohibition did not stop people from consuming alcohol, and it was repealed shortly after the Great Depression. After World War II, the Alcohol, Drug Abuse, and Mental Health Administration (ADAMHA) and the National Institute of Alcohol Abuse and Alcoholism (NIAAA) were developed. A primary goal of the organizations is to provide knowledge about the effects of alcohol on one's health and to improve the diagnosis, prevention, and treatment of alcohol-related problems, such as AUD.

**Alcohol Content of Beverages**

There is a wide range of alcohol content in alcoholic drinks; for instance, beer contains between 4% and 7% alcohol, while vodka contains between 40% and 50%. In the United States, one “standard” drink contains around 0.6 ounces of alcohol, equivalent to 14 grams of alcohol per drink. The percentage of the pure alcohol content is expressed by alcohol by volume (alc/vol), which varies across different beverage types. In Figure 6, the illustration provides examples of one standard drink across several types of alcoholic beverages.
Pharmacokinetics of Alcohol (Ethanol)

The psychoactive substance such as alcohol is among the oldest and most commonly used recreational drugs, causing various physiological effects. Alcohol is a weakly polar, aliphatic hydrocarbon soluble in water and lipid, influencing this drug's pharmacokinetics once consumed. Multiple factors, including absorption, distribution, and elimination, influence the pharmacokinetics of ethanol. The pharmacokinetics of ethanol is affected by many factors, including the type of alcohol consumed, gender, age, body weight, and metabolism. Like other drugs, alcohol is absorbed by the stomach and the small intestines and transported to the liver, going through first-pass metabolism before being distributed to other areas of the body. Studies have shown that 95% of the alcohol ingested is metabolized through enzymatic oxidation in the liver, and the remaining is excreted through sweat, urine, and breath. The two hepatic enzymes that
play a role in the enzymatic elimination process are alcohol dehydrogenase (ADH) and cytochrome (CYP2E1). Furthermore, the alcohol concentration contributes to the kinetics of alcohol metabolism as it initially undergoes a first-order Michaelis-Menten reaction before undergoing a zero-order reaction. \(^{[59,60]}\)

**Neurotransmitters & Alcohol**

Several neurotransmitters, such as dopamine, serotonin, glutamate, gamma-aminobutyric acid (GABA), opioid peptides, and adenosine, are involved in alcohol research. These neurotransmitters fall into three categories: excitatory neurotransmitters, which activate the postsynaptic cell; inhibitory neurotransmitters; which depress the postsynaptic cell's activity; and neuromodulators; which modify the postsynaptic cell’s response to other neurotransmitters. Each neurotransmitter inhibits different circuits, which determine mood, activity, and the behaviors that may come with alcoholism. Among these, dopamine is the neuromodulator that stimulates the transmission of motivational stimuli. The nucleus accumbens (NAc) is the brain region responsible for producing the initial reward signal. Studies have shown that once someone becomes addicted, the repetitive intake of the drug makes it up to the dorsal striatum, which is most commonly involved in habitual behaviors. Additionally, there is an increase in dopamine release in the NAc, even with low alcohol consumption. The release of dopamine contributes to the rewarding effects of alcohol, increasing the craving and desire to consume alcohol. \(^{[61]}\)
**Alcohol Use Disorder (AUD)**

An individual with AUD abuses alcohol or becomes dependent on it, despite its adverse effects, and it is considered a chronic brain disease. Those with AUD can fall into either mild, moderate, or severe addiction groups. Moreover, some individuals are still functional despite having an AUD, which depends on their severity. Individuals are more likely to experience relapse due to the changes caused by alcohol in their brains. Over time, binge drinking, and heavy alcohol consumption increase the risk of developing AUD. \[62,63\] According to the National Institute of Alcohol Abuse and Alcoholism, a national survey found that young adults aged 26 and older began drinking before the age of 15 and were five times more likely to develop an AUD than those who waited until the legal age of drinking. Individuals' drinking patterns can also be influenced by their genetics and environment. Lastly, mental health and trauma are also associated with AUD risk. It is common for people with psychiatric conditions such as depression, post-traumatic stress disorder, and attention deficit hyperactivity disorder to misuse or abuse alcohol. \[64\]

**DSM-5 Diagnosis of AUD**

Similar to other disorders, AUD is a problematic concern of chronically consuming alcohol which clinically leads to significant impairments or distress. Therefore, the DSM-5 created a list indicating that a patient showing at least two of the following occurring within 12 months could be diagnosed with AUD: \[65\]

1. Consuming alcohol frequently in large amounts over a long period

2. Having a persistent desire or unsuccessful attempts to reduce consumption
3. Spending a lot of time in activities involving alcohol or recovering from its effects

4. Craving alcohol and having a strong desire for it

5. Failing obligations at work, school, or home due to recurrent alcohol use

6. Continuous alcohol use, regardless of social and interpersonal problems caused by the effects of alcohol

7. Reducing important social, occupational, and recreational activities due to alcohol use

8. Partaking in activities that consume alcohol in physically hazardous situations

9. Alcohol use continues despite someone having a physical or psychological problem caused by alcohol

10. Acquiring a tolerance to alcohol can be defined by:

   a. The need to increase the amount of alcohol use to achieve intoxication or desired effects

   b. Having a diminished effect with continued use of the same amount of alcohol

11. Alcohol causes withdrawal effects

**Pharmacotherapies for AUD**

In American culture, alcoholism is a significant medical and social issue, causing more than 88,000 deaths yearly and costing more than $250 billion. The goal of AUD
treatments is to help patients quit drinking alcohol or reduce their consumption of alcohol in general. Furthermore, psychological, social, and pharmaceutical treatments have effectively reduced alcohol consumption in individuals. Even though there have been successful cases, the available treatments are ineffective for all patients. Thus, researchers are trying to find new pharmacotherapeutic approaches to aid individuals with this disorder.\textsuperscript{[65]}

\textit{Disulfiram}

The FDA approved disulfiram in the early 1950s as one of three medications to treat AUD. This drug acts as an acetaldehyde dehydrogenase inhibitor, therefore preventing the metabolism of acetaldehyde into acetic acid. Inhibiting aldehyde dehydrogenases causes acetaldehyde to accumulate in the blood with the consumption of alcohol, increasing unpleasant symptoms of skin flushing, tachycardia, hypotension, shortness of breath, nausea, and vomiting.\textsuperscript{[66]} Although this treatment has been on the market for over 60 years to treat AUD, clinical studies have revealed mixed results with the use of disulfiram. One of the most significant studies conducted with disulfiram used 605 male veterans with AUD and showed no significant difference between the different groups in the trial. The male veterans were divided into three groups, receiving either a therapeutic dose of disulfiram, a placebo dose of disulfiram, or a vitamin for a year. Disulfiram has a risk profile associated with some adverse effects that include death; however, some less severe side effects include headaches, drowsiness, tiredness, and halitosis, known as a metallic taste. In order to remain abstinent while on this medication, patients must have a good support system and self-discipline.\textsuperscript{[67,68]}
**Naltrexone**

The drug naltrexone, also known as Revia, is used to treat alcohol and opioid use disorder pharmacologically as a mu-opioid antagonist. This drug acts by reducing the cravings and the feelings of euphoria connected with SUDs. Naltrexone was developed in 1963 and patented in 1967. However, it was not until 1984 that naltrexone received its final approval for medicinal use in the United States, and 1994 for the treatment of alcohol dependence. The injectable version of naltrexone, marketed as Vivitrol™, was then approved and available for use in 2006. \(^{[67,69]}\)

**Figure 7 \[^{[107]}\]**

**Structure of Disulfiram**

![Structure of Disulfiram](image)

**Acamprosate**

Acamprosate, or N-acetyl homotaurine, is an N-methyl-D-aspartate (NMDA) receptor modulator approved by the FDA as a pharmacological treatment for AUD. This drug has been used in Europe as a treatment for alcohol dependence since 1989, and in
2004 it became the 3rd approved drug for alcohol treatment in the United States. Acamprosate is the structural analog of $\gamma$-aminobutyric acid (GABA). The mechanism of action of this drug is still unclear. However, it is thought to decrease alcohol intake by affecting calcium channels and modifying transmission along GABA and glutamine pathways in the brain. This process results in a decreased reinforcement of alcohol consumption and may also decrease the side effect of withdrawal cravings. In summary, acamprosate is not significantly more effective than disulfiram or naltrexone; additional studies have shown that combining acamprosate with other medications or psychosocial treatment has not been successful. [71,72]

Figure 8

Structure of Acamprosate

Beyond FDA-approved pharmacotherapies, behavioral therapies are available to treat AUD, including counseling, psychotherapy, and cognitive-behavioral therapy. Many communities also offer support groups, such as Alcoholics Anonymous (AA), to support
sobriety. These non-medications are either used alone or in combination with medications. \[64,74]\n
**Research Goal**

Millions of individuals worldwide are affected by AUD; the estimated 12-month prevalence and lifetime prevalence of DSM-5 criteria AUD among US adults was 13.9% and 29.1% between 2012-2013. In addition, AUD has been associated with substantial morbidity and mortality documented in New Jersey, with more than 1,700 deaths due to excessive alcohol consumption. Current treatments for AUD include naltrexone, acamprosate, and disulfiram which have only shown to be partially effective. Over the years, researchers have established the need to develop new pharmacotherapeutic treatments for patients with alcohol addictions. There have been varying results across patients on treatment for AUD, showing that only 16% of patients achieve abstinence. Another issue with AUD is its heterogeneity: not all medications will work for all patients. The development of new effective and diverse pharmacotherapy options will enhance the overall lifestyle of individuals during their treatment sessions. Due to the complexity of the neurobiological process that occurs in patients with AUD, it has been challenging to find new effective treatments.\[75]\n
The dopamine D3R is expressed in the areas of the brain associated with compulsive drug-taking and -seeking behaviors. A great deal of research has been conducted on D3 receptors and their role in modulating drug reward. Behavioral studies have shown that rodent models of SUDs, D3R selective antagonists, and partial agonists.
reduce self-administration and reinstatement of various drugs such as psychostimulants, opioids, and ethanol.\textsuperscript{[76]}

The unique pharmacology of cariprazine suggests that it may be suitable for treating ethanol-mediated behaviors as it has a high affinity for the dopamine D\textsubscript{3}R. Therefore, we hypothesized that if cariprazine can reduce ethanol intake, it could indicate that cariprazine, likely through D\textsubscript{3}R antagonism, can serve as a potential pharmacotherapeutic for AUD.\textsuperscript{[76]} In this study, cariprazine was evaluated for its effects on operant alcohol self-administration in mice. Our preclinical studies can later expand to evaluate cariprazine in combination with other FDA-approved drugs in animal models of AUD.
Chapter 2

Assessing the Possible Synergistic Effects of Morphine and MP-III-024 Co-Administration on Food Self-Administration, Open Field Behavior, Conditioned Place Preference, and Analgesic Tolerance

Introduction

The term "pain" describes any unpleasant sensation associated with actual or potential tissue damage. In 2019, the National Health Interview Survey (NHIS) reported that more than one in five adults in America suffers from chronic pain. Opioids are a class of drugs originally derived from the opium poppy plant, papaver somniferum, potent analgesics for pain management. Opioids activate four different opioid receptors found in the CNS and PNS. The four receptors are mu (µ), kappa (κ), delta (δ), and nociceptin/ orphanin FQ (NOP/FQ) opioid receptors. As a result of the activation of µ-opioid receptors, therapeutic relief is achieved, but some of the side effects of long-term use include tolerance, constipation, respiratory depression, and abuse potential. While opioid analgesics effectively treat acute and chronic pain, their undesirable side effects limit their therapeutic effectiveness. To provide patients with safer analgesic options, it is in demand to identify new pharmacotherapeutic strategies to treat pain.

γ-Aminobutyric acid (GABA) is a major inhibitory amino acid neurotransmitter in the CNS. GABA signaling is mediated by two receptor subtypes, ionotropic GABA\textsubscript{A} and metabotropic GABA\textsubscript{B}. Pentameric GABA\textsubscript{A} receptors include different isoforms, including different mixes of α, β, and γ, subunits, each containing different signaling and expression patterns across cell types. The effects of various GABA\textsubscript{A} positive
allosteric modulators (PAMs) are determined by the presence of α subunits α1, α2, α3, or α5. Benzodiazepines bind between α/γ GABA sites acting as PAMs thereby enhance GABA-induced chloride ion flux leading to hyperpolarization. This hyperpolarization of the cell prevents the further release of excitatory neurotransmitters. Studies have revealed that antinociception can be achieved through selective enhancement of GABA_A receptors via PAMs, including via benzodiazepines. GABA_A receptors containing α1 subunits, the subunit most associated with the sedative effects of benzodiazepines in addition to the effects related to abuse and physical dependence, are located more throughout the brain. In contrast, α2- and α3-containing GABA_A receptors are enriched in the spinal nociceptive circuits and are less associated with the negative side effects of GABA_A PAMs. Therefore, implementing a combination therapy of μ-opioid agonists with drugs targeting α2GABA_A and α3GABA_A receptors could be beneficial for treating pain. Combining a μ-opioid agonist with a benzodiazepine can allow medical personnel to prescribe lower doses of each constituent, thus reducing adverse effects and promoting synergism against a particular target. [51,97,99]

As reported in Fischer et al., 2017, the novel imidazodiazepine MP-III-024, a benzodiazepine-type compound with GABA_A PAM properties. MP-III-024 is functionally selective for α2GABA_A and α3GABA_A subtypes over the α1GABA_A and α5GABA_A subtypes. In addition to its high subtype selectivity, this compound exhibits a time course of action similar to morphine in rodent models, and negligible affinity for opioid receptors, making it an ideal candidate for combination therapy with morphine. [77,79]
Keck Lab alumnus Mohammad Atiqur conducted studies, published in 2021, investigating the interactive effects of μ-opioid agonist morphine and the α2/α3GABA_A receptor positive allosteric modulator, MP-III-024 in preclinical models of mechanical hyperalgesia and thermal nociception. These studies showed an interaction between α2/α3GABA_A receptors and the μ-opioid agonist receptor in rodent pain models, suggesting that combination therapy may be a therapeutic approach to treating pain-related disorders.

In the present study, we aimed to assess the possible synergistic effects of morphine and MP-III-024 in combination with several behavioral tests: food-self administration, open field behavior, conditioned place preference, and lastly, analgesic tolerance. The effect of different combinations of morphine and MP-III-024 were evaluated in male CD1 mice to determine the side effect profile of combining MP-III-024 and morphine in different behavioral assays.

**Interactive Effects of Morphine and MP-III-024 in Preclinical Models of Pain**

Rahman *et al.* in the Keck animal Behavioral lab, studied the antihyperalgesic and antinociceptive effects of morphine and MP-III-024 assessed individually and followed by fixed-ratio mixtures of morphine and MP-III-024. The two classical assays used in his studies were the von Frey test, used to evaluate inflammatory nociception, and a hot plate assay, used to evaluate thermogenic nociception. The two statistical analysis to evaluate the drug interactions in his experiments were isobolographic and dose-addition analyses.

[77]
For the von Frey studies, the mice were injected subcutaneously with zymosan A to provoke inflammation on their right hind paw; the left paw was left uninjected. Zymosan A causes an inflammatory response linked to its phagocytosis. The mechanical sensitivity test was assessed 24 hours after the zymosan A injection by applying von Frey filaments of increasing stiffness (5-26 g) to the mid-plantar surface of the hind paws until the filament bent. The maximal possible effect (%MPE) was determined using the following formula: 

\[ 100\% \times \frac{\text{post drug right paw threshold (g)} - \text{baseline right paw threshold (g)}}{\text{baseline left paw threshold (g)} - \text{baseline right paw threshold (g)}} \]

To test thermal nociception, the response time of each mouse was evaluated by recording the latency to lick or shuffle their hind paw(s) or jump from the hot plate surface a hot plate set at 56 °C ± 0.1 °C. A cutoff time of 20 seconds was used as the maximal response to prevent any damage to their paw tissue. The maximal possible effect (%MPE) was determined using the following formula:

\[ 100\% \times \frac{\text{post drug latency (s)} - \text{baseline latency (s)}}{20 - \text{baseline latency (s)}} \]

The von Frey assay revealed that morphine and MP-III-024 attenuated inflammatory pain. In Figure 9a, the %MPE of morphine and MP-III-024 for the mechanical test shows that each drug produced a dose- and time-dependent increase, indicating antinociceptive effects of the two drugs. Morphine alone and MP-III-024 alone were almost equipotent: the ED\textsubscript{50} (± SEM) of morphine was 10.72 (9.68–11.86) mg/kg and of MP-III-024 was 9.96 (8.81–11.26) mg/kg.

In the next test, morphine was given in combination with MP-III-024 at three ratios: 1.0:0.94, 1.0:2.8, and 1.0:0.31. These different ratios were derived using the log-linear interpolation by linear regression from Figure 9a. In Figure 9b, the relative
potencies of morphine and MP-III-024 show that with an increase in MP-III-024 there was a leftward shift in the graph, indicating a synergistic effect with the combination therapy. In Figure 10b, the isobolographic analysis of the drug combination for the mechanical hyperalgesia tests show that the 1.0:0.31 morphine and MP-III-024 mixture produced additive effects as the ED$_{50}$ values fell close to the line of additivity. In contrast the 1.0:0.94 ratio and the 1:2.8 ratio of morphine and MP-III-024 produced synergistic effects, as the values fell below the additivity line.
The Co-Administration of Morphine and MP-III-024 Attenuated Inflammatory Pain

Note. The effects of morphine, MP-III-024, and morphine and MP-III-024 at different ratios were assessed in a mechanical hyperalgesia assay. The x-axis represents the different doses assessed (1.0, 3.2, 10, and 32 mg/kg). The y-axis represents the calculated %MPE. A. Morphine and MP-III-024 alone were used as the baseline studies showing similar effects. B. Morphine and different mixed ratios of morphine and MP-III-024 (1.0:0.31, 1.0:0.94, and 1.0:2.8) show that an increase in drug concentration of MP-III-024 in the drug mix, the morphine dose effect curve showed a leftward shift, a synergistic effect. All results are presented as means ± SEM (n=8). Adapted from Rahman et al. (2021).
Note. A. This figure shows an example of two different drugs and the additivity line produced from the ED$_{50}$ values from each of the drugs. Dose pairs that fall below to the left of the additivity line suggests an ED$_{50}$ was reached with less quantities of the drugs. 

B. The Isobolograms of morphine and morphine and MP-III-024 at different mixed ratios (1.0:0.31, 1.0:0.94, and 1.0:2.8) were evaluated. All results are presented as means ± SEM (n=8). Modified from data presented in Rahman et al. (2021).

The hot plate assay revealed that the combination of morphine and MP-III-024 attenuated thermogenic pain. In Figure 11a, morphine shows to dose-dependently increases the animals’ latency to respond on the hotplate. The ED$_{50}$ (± SEM) of morphine was 14.98 (14.39-15.60) mg/kg and MP-III-024 produced no ED$_{50}$ value. Similarly, the same ratios tested in the von Frey procedure were evaluated in the hot plate assay. In Figure 11b, we see the same pattern of each of the different ratios assessed of morphine
and MP-III-024 1.0:0.31, 1.0:0.94, and 1.0:2.8) increased antinociception and MP-III-024 produced a left-ward shift in the morphine dose-effect curve. In Figure 12b, the isobolographic analysis of the isobolographic graphical analysis of the drug combinations for the thermal nociception assay reveals that each of the drug mixes tests produced supra-additive effects as the ED$_{50}$ values fell to the left of the line of additivity. [$^{[77]}$]

**Figure 11**

*The Co-Administration of Morphine and MP-III-024 Attenuated Thermogenic Pain*

*Note.* The effects of morphine, MP-III-024, and morphine and MP-III-024 at different ratios were assessed in a thermal sensitivity assay. The x-axis represents the different doses tested (1.0, 3.2, 10, and 32 mg/kg). The y-axis represents the calculated %MPE. A. The baseline studies reveal that morphine dose-dependently increased their latency to react, and MP-III-024 had no effect. B. Morphine and different mixed ratios of morphine and MP-III-024 (1.0:0.31, 1.0:0.94, and 1.0:2.8) show that with an increase in drug
concentration of MP-III-024 in the drug mix, the morphine dose effect curve showed a leftward shift, a synergistic effect. All results are presented as means ± SEM (n=8). Rahman et al. (2021).

**Figure 12**

*Isobolographic Hot Plate Analysis*

*Note.* **A.** This figure shows an example of two different drugs and the additivity line produced from the ED$_{50}$ values from each of the drugs. This isobolographic analysis only shows a vertical line in the case that only one drug, Drug A, produced an ED$_{50}$ value (i.e., Drug B was ineffective on its own). **B.** The Isobolograms of morphine and morphine and MP-III-024 at different mixed ratios (1.0:0.31, 1.0:0.94, and 1.0:2.8) were evaluated. All results are presented as means ± SEM (n=8). Modified from data presented in Rahman et al. (2021).
These findings indicate that adding an $\alpha_2/\alpha_3$GABA$_A$ PAM to a MOR agonist can potentiate opioid analgesic-like effects. The antinociceptive interactions depend on the relative proportions of each drug. These studies, however, did not test whether the drug combination also simultaneously increased effects unrelated to analgesia. The findings from this experiment, that the combination of morphine and MP-III-024 has a synergistic antinociceptive effect, led to the follow up question that we further investigated: **does co-administration of morphine and MP-III-024 produce a synergistic side effect profile?** The behavioral experiments to test our new hypothesis included operant conditioning, open field locomotor activity, conditioned place preference, and a chronic opioid-induced tolerance hot plate assay.

**Materials and Methods**

**Drugs**

**Morphine.** Morphine is a common analgesic alkaloid naturally derived from opium used to relieve pain. The scientific name of morphine is $(4R,4aR,7S,7aR,12bS)$-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7,9-diol. $^{[78]}$ The vehicle used to suspend this drug was 0.5% methylcellulose and 0.9% NaCl.
**Figure 13** [78]

*Structure of Morphine*

![Morphine Structure](image)

**MP-III-024.** MP-III-024 is a novel imidazodiazepine analog of benzodiazepines, known by methyl 8-ethynyl-6-(pyridin-2-yl)-4Hbenzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (MP-III-024). This drug was synthesized and received by the Department of Chemistry and Biochemistry at the University of Wisconsin-Milwaukee. This drug was suspended in 0.5% methylcellulose and 0.9% NaCl. It is a α2/α3GABA<sub>A</sub>-receptor-selective positive allosteric modulator (PAM) with a high subtype selectivity for opioid receptors. [79]
Animals

Adult male drug-naive CD-1 mice weighing 30-45 grams arrived from Charles River Laboratories. CD-1 mice are suitable for behavioral studies, mainly when testing different drug treatments. Upon arrival at the Cooper Medical School of Rowan University (CMSRU) vivarium, mice were grouped housed in standard Plexiglass cages in a room maintained on a 12-h light/dark cycle. The mice had continuous access to food and water throughout certain studies and habituated 2 weeks before any experimental procedure. Animals used for the following experiments were cared for per the guidelines of Rowan University's Institutional Animal Care and Use Committee.
Behavioral Assays

Operant Conditioning

Operant conditioning, originally developed by behaviorist B.F. Skinner, is a method that allows researchers to train a particular animal to perform specific tasks in response to specific stimuli. [81] In Figure 15, the self-administration operant chamber is displayed, showing that it’s equipped with a left nose poke associated with a correct response and a right response hole associated with an incorrect response. A correct response allows animals to receive positive reinforcement in the form of food or other rewards, and in some studies, they will also receive negative reinforcement. [81] During the training sessions, mice were trained to nose poke using a fixed ratio to obtain a programmed reward known as the FR system. Starting with a FR 1, one reward per nose poke, we gradually increase to a FR 4, one reward per four nose pokes. Each response hole is equipped with a stimulus light, a ventilated fan, and a syringe pump that administers the reward. The chambers also contain a reward receptacle. Prior to the experiment, a food restriction was necessary to enhance exploratory behavior, speeding up the process of operant training.
**Figure 15**

*Self-Administration Chamber*

![Self-Administration Chamber](image)

*Note.* This is the self-administration chamber that the mice are trained in. The chamber is equipped with a reward receptacle and two areas to nose poke; a correct (left) and incorrect (right) poke pole. With a correct nose poke, mice can consume their reward through the receptacle.

**Open Field**

An open field ANY-maze is used to analyze locomotor activity in animal models treated with a specific treatment. [82] **Figure 16** displays the open field chamber and program layout; each chamber measured approximately 40 × 40 × 35 cm and contained high walls to prevent mice from escaping their testing apparatus. The floor inserts were gray and had no gridlines. An overhead camera is placed above each chamber to detect...
the locomotion of each rodent using the ANY-maze. This camera helps distinguish between the time spent in the center and the perimeter of the enclosure. Different zones are set up before the experiment to set up a score of where the animal spends most of their time.

**Figure 16**

*Open Field Chambers*

*Note.* Open field chambers from Keck Animal Behavior Lab. **B.** Open field layout using the ANY-maze program, with a positioned camera above each chamber. The chamber is divided into 16 squares. The outer perimeter consisted of 12 squares and the 4 inner squares represented the chambers central region. The camera is used as a tracker to
collect the movement in the chambers to compare the time spent in the perimeter square versus the inner squares after drug administration.

**Conditioned Place Preference**

The conditioned place preference paradigm (CPP) is a behavioral test commonly used to study the rewarding properties of drugs. Repeated drug exposure is paired with a specific environment, which results in a Pavlovian association between the subjective drug effects and the environment. Figure 17 shows the CPP apparatus used in our behavioral studies. The three-compartment chamber contains different characteristics. The left chamber is white, while the right chamber is black, paired with different stainless steel grated floors. The middle compartment that separated the two chambers was gray with smooth flooring and was not associated with any drug pairing. During experimental training days, mice were injected with either the drug of interest or the vehicle. Each group received a training compartment-training dose combination and a matching compartment-vehicle dose combination. During the acquisition of CPP, animals were trained for 10 days and performed a final preference test on the 11th day. A CPP is found in animals that spend more time on the drug-paired versus the vehicle-paired side. When animals spend more time on the vehicle side, it is considered conditioned place aversion (CPA). It has been studied that abused drugs produce CPP, and drugs that elicit aversive effects produce CPA.  

[83]
Note. The conditioned place preference chamber consists of a white compartment on the left side and a black compartment on the right side. During experimental procedures, each side is paired with the drug of interest or the vehicle. The gray compartment located in the center is the neutral compartment, where the gates connecting the two compartments are closed during training days.

Hot Plate Assay

The hot plate assay is one of the oldest and most commonly used tests to assess nociception. Eddy and Leimbach proposed this behavioral assay for nociception in 1953.
and categorized the different reactions: nocifensive behaviors, such as jumping and hind paw-licking, occurred after noxious thermal stimulus. Licking is classified as a rapid response to painful thermal stimuli and an indicator of a nociceptive threshold. [51,84] Jumping represents an elaborated response to painful thermal stimuli, wherein the rodent expresses an emotional desire to escape. When testing for thermal pain sensitivity, the mice are placed on the hot plate (56.0 ± 0.1 °C), shown in Figure 18, and observers monitor for behavioral changes such as paw-licking, paw fluttering, and jumping. Drugs that alter the nociceptive threshold either increase the latency to react, an antinociceptive effect, or decrease it, known as a hyperalgesic effect. [85]
**Figure 18**

*Hot Plate Apparatus*

*Note.* The hot plate apparatus is set to 56.0 ± 0.1 °C. After the mice have received their treatment and are ready for testing, they are placed on the apparatus until a nocifensive reaction is observed (e.g., paw-licking, fluttering, and jumping).

**Von Frey Assay**

The physiologist Maximilian von Frey developed von Frey’s test to examine mechanical allodynia in mice, pain caused by an ordinarily painless stimulus. [86] The Von Frey assay aims to assess tolerance in our behavioral models. Animals are individually placed in small cages with a mesh bottom as shown in Figure 19. A filament
is applied perpendicularly to the plantar surface of the hind paw at a constant force under a reaction. The filaments used in this experiment vary in thickness usually (5-26 g). It is essential to acclimate the animals to the apparatus to ensure that the exploratory behaviors are not understood as positive responses.\[86\]

**Figure 19**

*Von Frey Apparatus*

*Note.* Mice are separated into their own compartment on an elevated wire grid. Prior to experiments the animals are habituated for a few hours. Different sized filaments (5-26 g) are placed onto the plantar surface of the paw increasing the pressure until the filament bends. A positive response is a clear paw withdrawal, shaking, or licking. A negative response is no response.
Experimental Procedure

Food Self-Administration Procedure

It is known that \( \mu \) opioid receptor agonists are behaviorally disruptive in operant tasks. The food self-administration procedure was conducted to determine whether the co-administration of MP-III-024 with morphine will enhance the behavioral disruption of morphine. Over two months, eight CD-1 male mice were trained to self-administer a solution containing 50% Vanilla Ensure and 50% water. The group sizes for these tests were determined from studies evaluating preliminary data from our lab and published literature. We conducted food self-administration studies seven days a week, to train the mice to nose poke to receive food rewards. Following each 120-minute training session, the animals were allowed to eat for an hour before fasting approximately 20-21 hours until their next experimental day. The eight mice initially began at an FR of 1 until they reached consistent responses at an FR of 4 and were ready to be tested with morphine and MP-III-024. Using a Latin square design as shown in Table 1, animals were injected on designated injection days with either morphine, MP-III-024, or a combination of morphine + MP-III-024 at different ratios. The different ratio doses evaluated are displayed in Table 2. A Latin square design is often used in animal experiments to minimize statistical variability. Therefore, at the end of this experiment each animal received one of each drug and vehicle dose. The drug was administered via intraperitoneal (i.p.) injections at a volume of 10 ml/kg. The mice were weighed every morning prior to making the appropriate injection volume. After receiving their injection, they were placed in their operant chambers for testing. As a result of this study, we can
evaluate the effects of morphine and a combination of morphine and MP-III-024 on food self-administration.

Table 1

*Morphine and MP-III-024 Drug Dosing: Latin Square Design*

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1A1</td>
<td>Vehicle</td>
<td>32 mg/kg</td>
<td>10 mg/kg</td>
<td>3.2 mg/kg</td>
</tr>
<tr>
<td>C1A2</td>
<td>3.2 mg/kg</td>
<td>Vehicle</td>
<td>32 mg/kg</td>
<td>10 mg/kg</td>
</tr>
<tr>
<td>C1A3</td>
<td>10 mg/kg</td>
<td>3.2 mg/kg</td>
<td>Vehicle</td>
<td>32 mg/kg</td>
</tr>
<tr>
<td>C1A4</td>
<td>32 mg/kg</td>
<td>10 mg/kg</td>
<td>3.2 mg/kg</td>
<td>Vehicle</td>
</tr>
<tr>
<td>C2A1</td>
<td>Vehicle</td>
<td>32 mg/kg</td>
<td>10 mg/kg</td>
<td>3.2 mg/kg</td>
</tr>
<tr>
<td>C2A2</td>
<td>3.2 mg/kg</td>
<td>Vehicle</td>
<td>32 mg/kg</td>
<td>10 mg/kg</td>
</tr>
<tr>
<td>C2A3</td>
<td>10 mg/kg</td>
<td>3.2 mg/kg</td>
<td>Vehicle</td>
<td>32 mg/kg</td>
</tr>
<tr>
<td>C2A4</td>
<td>32 mg/kg</td>
<td>10 mg/kg</td>
<td>3.2 mg/kg</td>
<td>Vehicle</td>
</tr>
</tbody>
</table>

*Note.* This table shows the Latin square design used in the food self-administration studies. The experimental testing days were every other day. On the days when they do not receive an injection, they underwent their regular testing sessions. After the 9th day every animal received an injection of each of the doses (3.2, 10, and 32 mg/kg of morphine and a combination of morphine and MP-III-024 at 3.2, 10, 32 mg/kg).
Table 2

Food Self-Administration Drug Dosing Ratios

<table>
<thead>
<tr>
<th>Ratio → 1.0:0.31</th>
<th>Ratio → 1.0:0.94</th>
<th>Ratio → 1.0:2.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>MP-III-024</td>
<td>Morphine</td>
</tr>
<tr>
<td>1.0 mg/kg</td>
<td>0.31 mg/kg</td>
<td>1.0 mg/kg</td>
</tr>
<tr>
<td>2.2 mg/kg</td>
<td>0.682 mg/kg</td>
<td>2.2 mg/kg</td>
</tr>
<tr>
<td>6.8 mg/kg</td>
<td>2.108 mg/kg</td>
<td>6.8 mg/kg</td>
</tr>
<tr>
<td>8.0 mg/kg</td>
<td>2.48 mg/kg</td>
<td>8.0 mg/kg</td>
</tr>
<tr>
<td>14.0 mg/kg</td>
<td>4.34 mg/kg</td>
<td>14.0 mg/kg</td>
</tr>
</tbody>
</table>

Note. This table demonstrates the drug dosing for the food self-administration studies for the combination therapy of morphine and MP-III-024 at the three tested ratios (1:0.31, 1.0:0.94, and 1.0:2.8). The different ratios were derived using the log-linear interpolation by linear regression from Rahman et al.’s dose-response curve results for morphine and MP-III-024. [77]

Open Field Procedure

The purpose of an open field locomotor tests is to measure the neurochemical effects that morphine, MP-III-024 and a combination of morphine and MP-III-024 have in rodent models. Additionally, it is known that µ opioid receptor agonists induce hyperlocomotion in rodents. Similar to the food self-administration procedure, eight CD-1 male mice were used in the open field study. The group sizes for these tests were determined from studies evaluating preliminary data from our lab and published literature. On the first day, all eight mice were allowed to explore the open field chamber.
for 15 minutes to become accustomed to the environment. On the second day, all 8 mice were given 6 i.p. injections of vehicle (0.5% methylcellulose in 0.9% saline). After each injection, a 30-minute exploration of the open field chamber was allowed to monitor the mice's locomotor activity. In these studies, activity was monitored by ANY-maze tracking software containing cameras above each chamber. The third day of testing consisted of 6 i.p. injections of incremental doses of morphine, MP-III-024, or a combination of morphine + MP-III-024 at different ratios. Each animal was given a cumulative dose of 32 mg/kg, as shown in Table 3. Additionally, the 3 different ratios tested for the open field assessment are displayed in Table 4.
Table 3

*Open Field Test Drug Doses*

<table>
<thead>
<tr>
<th>Drug Dose</th>
<th>Cumulative Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acclimation</td>
<td>No injection</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Vehicle</td>
</tr>
<tr>
<td>1.0 mg/kg</td>
<td>1.0 mg/kg</td>
</tr>
<tr>
<td>2.2 mg/kg</td>
<td>3.2 mg/kg</td>
</tr>
<tr>
<td>6.0 mg/kg</td>
<td>6.8 mg/kg</td>
</tr>
<tr>
<td>8.0 mg/kg</td>
<td>14.0 mg/kg</td>
</tr>
<tr>
<td>14.0 mg/kg</td>
<td>32.0 mg/kg</td>
</tr>
</tbody>
</table>

*Note.* The open field procedure began with an acclimation period of 15 minutes prior to testing the first dose of 1.0 mg/kg. After each injection the animals were placed in their testing chambers for 30 minutes until their next injection. Each animal received a cumulative dose of 32 mg/kg of either morphine, MP-III-024, or a combination of morphine and MP-III-024.
Table 4

Open Field Drug Dosing Ratios

<table>
<thead>
<tr>
<th>Ratio → 1.0:0.31</th>
<th>Ratio → 1.0:0.94</th>
<th>Ratio → 1.0:2.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
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</tr>
<tr>
<td>1.0 mg/kg</td>
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</tr>
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<td>8.0 mg/kg</td>
<td>2.48 mg/kg</td>
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</tr>
<tr>
<td>14.0 mg/kg</td>
<td>4.34 mg/kg</td>
<td>14.0 mg/kg</td>
</tr>
</tbody>
</table>

Note. This table demonstrates the different drug mixtures for the combination group of morphine and MP-III-024. The different drug mixtures of morphine and MP-III-024 tested were 1.0:0.31, 1.0:0.94, and 1.0:2.8. The ratios were derived using the log-linear interpolation by linear regression from Rahman et al.’s dose-response curve results for morphine and MP-III-024. [77]

Conditioned Place Preference Procedure

To further evaluate the effects of this combination therapy, we wanted to determine whether MP-III-024 causes any abuse liability on morphine. This classical assay tests the addictive liability of drugs of abuse, here we evaluate whether MP-III-024 will affect the liability of morphine, and if MP-III-024 has any abuse liability on its own. An initial preference for each animal is determined prior to conditioning or training drugs of interest. This initial preference is essential to eliminate biased factors in the
experimental procedure. This initial preference was measured by allowing free access to the apparatus for 30 minutes. The ratio of time spent in each compartment was then evaluated to randomly assign each animal a drug-paired side and a vehicle-paired side.

Place preference was explored at 3, 10, 17.8, and 32 mg/kg of morphine, MP-III-024, or a combination of morphine and MP-III-024 at the 1.0:0.94 ratio. The initial design called for 12 animals per group, and a total of 120 animals. This group size was determined from other studies that evaluate preliminary data from our lab and other literature. However, some groups ended up with substantially more or less than 12 due to removal of animals with initial side preferences greater than 65% as well as planning errors. With only four CPP chambers, these tests were conducted over a long period of time and the number of subjects in each category was recorded erroneously until our final analyses. Final group numbers are shown in Table 5. In total there were 117 animals assessed at the end of the study.

During their conditioning sessions, the mice were restricted to the appropriate compartments following drug or vehicle i.p. injections, alternating exposures daily for 10 training sessions. The final preference is taken on the 11th day; mice are given free access to the entire apparatus without an injection. Here we can evaluate whether MP-III-024 causes a place preference on its own (indicating potential abuse liability) or enhances morphine-induced place preference (indicating an increased risk of opioid-driven abuse liability).
Table 5

*Conditioned Place Preference Drug Doses & Groups*

<table>
<thead>
<tr>
<th>Morphine</th>
<th>MP-III-024</th>
<th>Morphine + MP-III-024 (1:0.94)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mg/kg (n=14)</td>
<td>3 mg/kg (n=6)</td>
<td>3 mg/kg (n=10)</td>
</tr>
<tr>
<td>10 mg/kg (n=10)</td>
<td>10 mg/kg (n=10)</td>
<td>10 mg/kg (n=12)</td>
</tr>
<tr>
<td>17.8 mg/kg (n=13)</td>
<td>17.8 mg/kg (n=14)</td>
<td>17.8 mg/kg (n=10)</td>
</tr>
<tr>
<td></td>
<td>32 mg/kg (n=18)</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* This table shows each of the doses tested for morphine, MP-III-024, and morphin+MP-III-024 at the 1.0:0.94 ratio (3, 10, 17.8, and 32 mg/kg). The initial plan was to have a total of 12 animals per group, however these experiments were conducted at different times which led us to have more animals in certain groups and less in others. The animals were grouped after their first initial preference and randomly assigned a drug dose and pairing side.

*Morphine Tolerance Assay*

The hot plate procedure was evaluated using 16 male CD-1 mice, divided into two groups: one that tested only morphine (n=8), while the other tested a combination of morphine and MP-III-024 (n=8). The group sizes for these tests were determined from studies evaluating preliminary data from our lab and published literature. The experimental drug design in Figure 20, shows that two baselines were measured from each animal before beginning the experiment using a hot plate set to 56 ± 0.1 °C. On day 1, the mice underwent a cumulative dosing regimen of morphine at 0, 1, 3.2, 10, and 32
mg/kg, shown in Table 6. Immediately following the i.p. injection, mice were placed back in their cage immediately and placed on the hotplate after 30 minutes for testing. The latency of their reaction was recorded in seconds, having a maximum effect of 20 seconds. Throughout days 2-6, the mice had chronic treatment of 100 mg/kg morphine or a combination of 100 mg/kg morphine + 17.8mg/kg MP-III-024 at 10:00 am and 4:00 pm. On day 7, the mice followed the same procedure as day 1, except an additional dose of 68 mg/kg was administered to total a cumulative dose of 100 mg/kg.
**Figure 20**

*Tolerance Test Experimental Design*

<table>
<thead>
<tr>
<th>Day 1: Cumulative Morphine Dosing</th>
<th>Day 2-6 Chronic Morphine Exposure</th>
<th>Day 2-6 Chronic Morphine + MP-III-024 Exposure</th>
<th>Day 7: Cumulative Morphine Dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, 3.2, 10, and 32 mg/kg</td>
<td>100 mg/kg × 2 (n=8)</td>
<td>100 mg/kg morphine + 17.8 mg/kg MP-III-024 × 2 (n=8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0, 3.2, 10, 32, and 100 mg/kg</td>
</tr>
</tbody>
</table>

*Note.* Prior to beginning the experiment two baseline measures were taken from each mouse. On day 1 and day 7, 30 minutes after each injection, each mouse was placed on a hot plate set at 56.0 ± 0.1 °C. On days 2-6, eight animals received 100 mg/kg of morphine twice a day, at 10:00am and 4:00pm; and the other eight animals received a combination of morphine and MP-III-024, 100 mg/kg morphine + 17.8 mg/kg MP-III-024, at the same time.
### Table 6

*Tolerance Assay Experimental Drug Design*

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Morphine (n=16)</th>
<th>0, 1, 2.2, 6.8, 22 mg/kg (32 mg/kg total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2-6</td>
<td>Morphine (n=8)</td>
<td>100 mg/kg (morphine twice a day)</td>
</tr>
<tr>
<td></td>
<td>Morphine + MP-III-024 (n=8)</td>
<td>100 mg/kg + 17.8 mg/kg (morphine:MP-III-024 twice a day)</td>
</tr>
<tr>
<td>Day 7</td>
<td>Morphine (n=16)</td>
<td>0, 1, 2.2, 6.8, 22, 68 mg/kg (100 mg/kg total)</td>
</tr>
</tbody>
</table>

*Note.* This table demonstrates the experimental drug design for the tolerance test. On the first day all 16 animals were given successive injections of 0, 1, 2.2, 6.8, and 22 mg/kg morphine, a total cumulative dose of 32 mg/kg morphine. On days 2-6, animals were separated into two separate groups: 8 animals received 100 mg/kg morphine twice daily, and the other 8 received 100 mg/kg morphine + 17.8 mg/kg MP-III-024 twice daily. On day 7, all 16 animals were given successive injections of 0, 1, 2.2, 6.8, 22, and 68 mg/kg morphine, a total cumulative dose of 100 mg/kg morphine.
Data & Results

*Food Self-Administration Results*

**Figure 21**

*Food Self-Administration Rewards*

![Graph showing Food Self-Administration Rewards](image)

*Note.* The food self-administration graph shows the reward values tested in morphine alone and in different mixed ratios of morphine + MP-III-024 (1.0:0.94, 1.0:0.31, and 1.0:2.8). We want to evaluate whether MP-III-024 influences morphine’s behavioral effects. The x-axis represents the tested doses of either morphine alone or morphine + MP-III-024 (3.2, 10, or 32 mg/kg). The y-axis represents the number of earned rewards out of 100 maximum rewards. Two-way ANOVA revealed that morphine significantly reduced the number of earned palatable food rewards. However, the three drug doses...
tested (1.0:0.94, 1.0:0.31, and 1.0:2.8) did not significantly affect the earned rewards when comparing it to morphine alone. All results are presented as means ± SEM (n=8).

**Figure 22**

*Food Self-Administration Response Rates*

*Note.* This graph represents the food self-administration response rate values for morphine and the combination drug therapy of morphine and MP-III-024 at different mixed ratios (1.0:0.94, 1.0:0.31, and 1.0:2.8). We wanted to evaluate the effects of MP-III-024 to see if it would enhance or attenuate morphine’s behavioral effects. The x-axis represents the doses tested of either morphine alone or morphine + MP-III-024 (3.2, 10, or 32 mg/kg). The y-axis represents the animals’ response rates. A two-way ANOVA analysis revealed that morphine significantly reduced the animals’ operant responding, but the drug mixes tested (1.0:0.31, 1.0:0.94, and 1.0:2.8) did not significantly reduce that
animals’ response rate when comparing it to morphine alone. These results can lead us to conclude that when morphine is co-administered with MP-III-024 it has statistically indistinguishable effects from morphine alone; however, the addition of MP-III-024 to morphine does not make morphine more disruptive. This graph could also possibly reveal a slight rightward shift at the 1.0:0.94 ratio, concluding to have an anti-synergistic effect. All results are presented as means ± SEM (n=8).

From the data collected; Figure 21 shows that morphine dose-dependently disrupts operant response to palatable food used, vanilla ensure. Furthermore, when adding MP-III-024 to morphine at different ratios, we found that it did not make morphine more disruptive. However, at the 1.0:0.94 drug mixture of morphine and MP-III-024, we can observe a rightward shift. This restoration of a rightward shift is better observed in Figure 22, when comparing the animals’ response rates. MP-III-024 alone was not tested since our collaborator Fischer et al., 2017 performed behavioral tests on MP-III-024, revealing that MP-III-024 was ineffective across all the doses tested. They could not determine an ED₅₀ value for this compound, indicating that when given in combination with morphine, MP-III-024 may be beneficial in neutralizing the adverse side effects associated with drug types like morphine. In Figure 23, shows the control studies on MP-III-024, revealing no decrease in operant response rates at doses 3.2, 10, 32, and 100 mg/kg. The results of this study suggest that MP-III-024 can be used as a research tool for studying how benzodiazepines-like drugs influence rodent behavioral properties. The two-way ANOVA analysis revealed that the drug mixture at every tested ratio (1.0:0.31, 1.0:0.94, and 1.0:2.8) did not significantly reduce mice's motivation to
nose poke for palatable food rewards. The response rates are an important measure in these behavioral assays since they measure how fast or slow a mouse pokes its nose, which is more indicative of fundamental behavioral changes than incentives earned. From this we can conclude that the co-administration of morphine and MP-III-024 at the 1.0:0.94 ratio does not disrupt behavioral self-administration, suggesting that it may be subadditive or anti-synergistic.

**Figure 23**

*Effects of MP-III-024 on Food-Maintained Operant Responding*

*Note.* The effects of MP-III-024 on food-maintained operant behavior. These results show that MP-III-024 did not alter operant behavior up to 100 mg/kg. This data suggests that MP-III-024 can give antihyperalgesic effects while lacking sedative-like effects. Modified from data presented in Fischer *et al.* (2017).
**Open Field Locomotor Results**

**Figure 24**

*Open Field Locomotor Results*

**Note.** The open field graphs represent mouse locomotor activity during a cumulative dosing procedure. The x-axis represents the cumulative doses tested (3.2, 10, 18, and 32 mg/kg). The y-axis represents the distance the mice traveled in meters (m) during each training session. This assay tested morphine, MP-III-024, and different mixed ratios of morphine and MP-III-024 (1.0:0.31, 1.0:0.94, and 1.0:2.8). **A.** This figure shows the 5-min bins of each of the different groups tested. Morphine is shown to induce hyperlocomotive effects, in addition to the 1.0:2.8 and 1.0:0.31 ratio of morphine and MP-III-024 together. Additionally, MP-III-024 did not significantly affect locomotion. It is also seen that at the 1.0:0.94 ratio their locomotion was attenuated. **B.** This graph represents the averages of each of the animals’ 5-min bins; the Two-way ANOVA
showed no statistical significance between morphine alone and the 1.0:0.31 and 1.0:2.8 ratio mixtures). However, there is a statistical significance between morphine alone and the 1.0:0.94 ratio, meaning this dose has an anti-synergistic profile. All results are presented as means ± SEM (n=8 per group).

Figure 25

Comparison of Vehicle and MP-III-024 in Locomotor Activity

*Note.* This graph compares the locomotor activity of the vehicle and MP-III-024. The x-axis represents the cumulative doses given (3.2, 10, 18, and 32 mg/kg). When comparing the two, it can be concluded that there is no statistical significance between the locomotor activity of vehicle or MP-III-024. All results are presented as means ± SEM (n=8 per group).
Comparison of Morphine and the Different Drug Mixtures Tested

Note. An additional graph was analyzed comparing morphine alone against the 3 different ratios tests (1.0:0.94, 1.0:0.31, and 1.0:2.8) at each specific dose. The x-axis represents the cumulative drug dose the animals received every 30 minutes. (3.2, 10, 18, 32 mg/kg). The y-axis represents the distance the mice traveled in meters (m) during each training session. Two-way ANOVA revealed that the 1.0:2.8 ratio was significant from morphine at 18 and 32 mg/kg. Additionally, the 1.0:0.94 ratio significantly reduced hyperlocomotion activity at the 32 mg/kg when comparing it to morphine alone. All results are presented as means ± SEM (n=8 per group).

From the findings of the open field experiment, we can conclude that the co-administration of morphine and MP-III-024 do not have synergistic locomotor-enhancing
effects. In Figure 24a, we graphed the 5-minute bins of the locomotor activity of each animal; comparing each of the different groups tested morphine, MP-III-024, and combination of morphine and MP-III-024 at (1.0:0.31, 1.0:0.94, and 1.0:2.8). This graph shows that morphine-dose dependently increases locomotor activity. Moreover, MP-III-024 did not significantly affect locomotion. Fischer et al. also confirmed that MP-III-024 did not affect the rodent's locomotor activity. These findings reveal a heteroergic effect, in which morphine had an effect, and MP-III-024 was insufficient to provide an effect.

When comparing the different drug mixes, we see that the 1.0:0.94 ratio significantly attenuated morphine-induced hyperlocomotion. However, the animals that received 1.0:0.31 and 1.0:2.8 ratios of morphine and MP-III-024 had higher locomotor activity when compared to morphine alone. Ideally, it was expected for these two doses would fall below the hyperlocomotion induced by morphine alone. It is important to note that we tested the different drug mixtures at different times and the same group of animals were not used throughout the entire study. The first batch of animals that were received we used to test morphine, and the 1.0:0.94 drug mixture; we then received a different batch of animals to test the additional two drug mixtures. In Figure 24b, the 5-minute bins were averaged to evaluate the different groups that were tested. The two-way ANOVA analysis reveals no statistical significance between the morphine alone group and the 1.0:0.31 and 1.0:2.8 drug mixes. This analysis also reveals that there was a statistical significance between morphine alone and the 1.0:0.94 drug mixture, which can indicate an anti-synergistic effect. In Figure 25, we further evaluated the vehicle and MP-III-024 alone, to specifically determine whether MP-III-024 reduced their locomotion completely or if it followed the same pattern as the vehicle. When comparing
these two doses, there was no statistical significance between the locomotor activity of vehicle or MP-III-024. In Figure 26, we then compared morphine against the three drug mixes 1.0:0:94, 1.0:0.31, and 1.0:2.8. A two-way ANOVA revealed that the 1.0:2.8 drug mixture at 18 and 32 mg/kg was significant when comparing it to morphine. Additionally, the 1.0:0.94 drug mixture significantly reduced hyperlocomotion activity at 32 mg/kg when comparing it to morphine.

**Conditioned Place Preference Results**

**Figure 27**

**Conditioned Place Preference Results**
Note. Conditioned place preference was performed on morphine, MP-III-024, and in combination at the 1.0:0.94 ratio of morphine and MP-III-024. Here, we evaluate whether MP-III-024 affects the abuse liability of morphine. The one-way ANOVA test revealed multiple comparisons across the preference scores at different doses of morphine, MP-III-024, and 1.0:0.94 ratio of morphine and MP-III-024, revealing that there is only a significant difference between 3 mg/kg and 10 mg/kg of morphine with a (p = 0.0201). Additionally, the 1.0:0.94 ratio may possibly cause a right-ward shift in the curve, seeing that MP-III-024 does not induce CPP. All results are presented as means ± SEM.

The conditioned place preference results reveal that MP-III-024 does not enhance place preference for morphine. In Figure 27, when comparing the conditioned place preference of morphine, MP-III-024 and the combination of morphine and MP-III-024 at the 1.0:0.94 ratio; there was only a statistical significance in CPP at 10 mg/kg of morphine. The graph reveals that the peak for morphine alone is seen at 10 mg/kg, and the peak for the combination of morphine and MP-III-024 is seen at 17.8 mg/kg, indicating that with the presence of MP-III-024, it takes more morphine to induce CPP. Therefore, the right-ward shift could indicate a sub-additive effect (anti-synergistic) from the drug combination. Additionally, MP-III-024 alone did not induce CPP. Additionally, morphine alone produced no conditioned place preference.
**Hot Plate Assay Results**

**Figure 28**

*Development of Morphine Tolerance as Measured by the Hot Plate Assay*

*Note.* Chronic morphine induces tolerance to the drug’s analgesic effects. In order to assess whether co-administration of MP-III-024 alters the development of morphine tolerance, mice were treated with 5 days of chronic morphine (dosing), or morphine co-administered with MP-III-024 (dosing). **A.** Cumulative dosing of morphine before and
after the chronic treatments show a rightward shift of the morphine dose-effect curve in
the hot plate assay. **B.** Analysis of the individual morphine ED$_{50}$s shows that co-
administration of MP-III-024 did not enhance the development of morphine tolerance.
Mixed ANOVA evaluating chronic drug treatment (morphine or morphine/MP-III-024
coadministration) as between-subjects factor and individual morphine hot plate ED$_{50}$
values before and after chronic treatment as within-subjects factored revealed a
significant effect of the chronic morphine treatment ($F_{(1, 12)} = 24.75, P = 0.0003$) but no
effect of the absence or presence of MP-III-024 with the chronic morphine ($F_{(1, 12)} =
2.110, P = 0.1720$). All results are presented as means ± SEM (n=16).

The findings from the tolerance test show that there is no evidence that MP-III-
024 enhances morphine tolerance. **In Figure 28a,** we compared the initial and final test
for morphine alone and the mixed drug mixture of morphine and MP-III-024 at the
1.0:0.94 ratio. We can observe an apparent rightward shift of the morphine dose-effect
curve before and after their treatment. To better evaluate our results, in figure 28b, we
graphed the individual ED$_{50}$ values of each animal before and after their treatment. A
mixed ANOVA analysis revealed that there was significance in chronic drug exposure
but no specific effect on the different drugs. We compared multiple different t-tests; a
morphine-paired t-test, a combination-paired t-test, and an unpaired t-test; revealing that
MP-III-024 did not enhance the development of morphine tolerance.
Discussion

Opioids interact with opioid receptors in the body to produce analgesia, causing pleasurable feelings of euphoria. The downfall of opioid use is the severe side effects, including respiratory depression, the number one leading cause of death from opioid use, and its misuse and abuse. Our drug of interest, MP-III-024, enhances signaling at the GABA<sub>A</sub> receptors at the α2/α3 subunits. GABA<sub>A</sub> receptors can also achieve antinociception which is why we want to test it in combination with the MOR agonist morphine; to selectively target pain posing fewer side effects. Scientists may be concerned with the affects that combining a benzodiazepine-like drug with an opioid agonist may have, however our drug of interest differs from other benzodiazepines. Our collaborator Fischer et al., 2017 notes that GABA<sub>A</sub> receptors containing α1 subunits are located more throughout the CNS, which leads to an increase in the sedative effects of benzodiazepines in addition to the effects related to abuse and physical dependence. However, α2- and α3-containing GABA<sub>A</sub> receptors are commonly found in the spinal nociceptive circuits and do not appear to be associated with the negative side effects of PAMs. Therefore, this benzodiazepine-like drug could show promising results in dual-treatment with morphine. The interactive effects of drug mixtures depend on their relative proportions. As previously noted, Rahman et al. discovered that the 1.0:0.94 ratio of morphine and MP-III-024 had the most significant effect on antinociceptive synergy. Our food self-administration results show a possible sub-additive or anti-synergistic effect at the 1.0:0.94 ratio. Moreover, the locomotor results do not have synergetic locomotor-enhancing effects. We found that the combination of morphine and MP-III-024 also has a subadditive effect at the 1.0:0.94 ratio. We can propose from our results that morphine and MP-III-024 in combination are not universally synergistic; the
interactive effect of drug mixes depends on their relative proportion; hence, they may increase the therapeutic window between desirable and undesirable effects caused by opioids.

The conditioned place preference assay evaluates the psychoactive properties of morphine and MP-III-024 in rodents; to help determine if MP-III-024 causes any abuse liability on morphine or if MP-III-024 has any effect on its own. From a clinical standpoint, if the combination therapy produces a leftward and synergistic effect on pain, but a rightward antagonistic effect on locomotor, self-administration, and CPP assays, it can be a beneficial therapeutic therapy to implement. The goal is to find medications that increase the analgesic therapeutic window with a dual-pharmacology strategy. The results from the CPP studies we can conclude that in the presence of MP-III-024 more morphine is required to induce a preference. This method is ideal as it would decrease the amount of opioid dosage in medications in turn reducing the probability of patients developing an addiction.

The hot plate assay measure thermal nociception which can be used to evaluate tolerance. Here, we tested whether MP-III-024 has an effect on morphine tolerance. It is well known that tolerance is one of the side effects associated with chronic morphine exposure. The mice were treated with chronic exposure to either morphine or co-administration of morphine MP-III-024. Our findings conclude that MP-III-024 did not enhance morphine tolerance, thus a dual MOR-α2/α3GABA\A pharmacotherapy strategy could be beneficial in targeting analgesia with less secondary effects than taking opioids alone.
It is important to note that this study had some limitations; the first limitation was that only male mice participated in the experiments. Studies have shown a sex-mediated difference in opioid signaling and comparing the results of male and female mice would be interesting. Furthermore, for our open field tests we performed the experiment with two different groups of animals. Lastly, when conducting the tolerance test the animals were not randomized into different treatment groups within their housing unit, therefore this could have influenced their behavior.

Some follow-up studies include investigating whether the co-administration of MP-III-024 affects morphine-induced respiratory depression. Respiratory depression will be evaluated using plethysmography, which evaluates respiratory function. Respiratory dysfunction is the leading cause of death that comes with OUD. Another topic of interest is looking at the side effect of constipation and investigating other opioid receptors to see if this dual-pharmacology strategy is effective with other receptors.

The simultaneous use of opioids and benzodiazepine medications has been noted to have risks despite having limited data to conclude this. There is a fear that combining opioids with benzodiazepines can increase the risk of overdose due to sedation and suppression of breathing. However, studies have shown that there are diverse behavioral effects of benzodiazepine drugs which are reflected in different GABA<sub>α</sub> receptor subtypes. The precise role of each of the GABA<sub>α</sub> α-subunits in the antihyperalgesic properties of benzodiazepine drugs has not been fully evaluated due to limited available compounds for research. In order to limit the negative effects that co-administering opioids and benzodiazepines can have, it is important to research compounds that can specifically target selective α-subunits to limit some of the side
effects that come with benzodiazepine drugs. Thus, MP-III-024 is a benzodiazepine type drug which differs from other compounds since it includes an imidazole. \[79\]
Chapter 3

The Effects of the Dopamine D3 Receptor Antagonist Cariprazine on Alcohol and Palatable Food Self-Administration

Introduction

AUD is one of many types of SUDs, a disorder of the brain that impairs control over alcohol use regardless of the adverse effects it may cause. It is important to note that a lack of self-discipline does not cause AUD; it is an inherited condition resulting in a craving for alcohol and an inability to control consumption, requiring more significant amounts of alcohol. Alcoholism and the prevalence of alcohol use continue to increase in the United States. The 2019 National Survey on Drug Use and Health (NSDUH) reported that 85.6% of people aged 18 and older consumed alcohol in the past year and 65.9% in the previous month. Additionally, 54.9% of men and 51.0% of women reported drinking in the past month. According to the NSDUH, 14.5 million people aged 12 and over have AUD. Currently, pharmacotherapeutic treatments for AUD include naltrexone, acamprosate, and disulfiram, but they are clinically effective for only a small portion of the AUD population. Based on these statistics and currently available treatments, a clear need exists to develop better and new pharmacotherapeutic methods for treating AUD patients.

Several addictive drugs, including alcohol, increase dopamine (DA) signaling in brain circuits controlling reward, emotion, cognition, and motivation. The molecular targets of addictive drugs vary; however, they all enhance DA signaling in the ventral striatum, particularly in the nucleus accumbens, activating neural circuitry, which
mediates reward responses to natural external stimuli such as food and sex.\textsuperscript{[76]} Dopamine exerts its effects through five subtypes of receptors (D₁R-D₅R), but the D3 receptor (D₃R) has demonstrated a crucial role in developing medications to treat substance abuse disorders. Various D₃R-selective compounds with high affinity and varying efficacy have been developed and applied to in vivo drug abuse models. Several studies have explored the involvement of D₃R in ethanol-drinking paradigms; however, their precise role remains unclear.\textsuperscript{[76]} Leggio \textit{et al.} reported that the D₃R gene deletion or D₃R pharmacological blockade inhibited ethanol intake. D₃R antagonists and partial agonists are promising drug classes in rodent models of relapse-like behavior, namely stress-, drug-, and cue-induced reinstatement of drug seeking.\textsuperscript{[89]} Additionally, Rice \textit{et al.} study revealed that a selective D₃R antagonist drug significantly reduced the consumption of ethanol in restricted-access binge-drinking assay in mice models.\textsuperscript{[103]} However, the translation of these preclinical studies has been relatively unsuccessful to-date, which is why new treatments are needed for AUD patients.

Cariprazine (Vraylar\textsuperscript{™}) is a medication known to treat symptoms for psychotic disorders such as schizophrenia, manic disorder, and bipolar disorder. Cariprazine (Vraylar\textsuperscript{TM}) is a D₃R preferential ligand with low intrinsic efficacy at D₃R, attenuating D₂R- like signaling which works by eliminating the side effects associated with first-generation antipsychotics.\textsuperscript{[91,92]} Based on the evidence that cariprazine has a D₃R-preferential signaling profile and is currently FDA-approved and available for clinical use, it could be a successful “low-hanging fruit” treatment option for patients suffering from AUD and other substance use disorders. Román \textit{et al.} studied animal models with
cocaine use disorder and reported that cariprazine reduces cocaine self-administration and cocaine-seeking behavior.\textsuperscript{[90]}

These findings led to the idea of testing cariprazine on animal models of AUD, which have yet to be evaluated. We hypothesized that if cariprazine, a D\textsubscript{3}-preferring dopamine D\textsubscript{3}R/D\textsubscript{2}R partial agonist, can alter alcohol self-administration in male and female C57Bl/6 mice it could serve as a potential pharmacotherapeutic for AUD, and other addictions. Future studies will also evaluate whether cariprazine can enhance the efficacy of current AUD treatments like naltrexone and acamprosate if given in combination. The benefits of drug combinations include effectively administering the drugs at lower doses, decreasing undesirable side effects, and targeting multiple pathways that mediate alcohol-seeking behaviors.

**Materials and Methods**

**Drug**

**Cariprazine.** The drug of interest, cariprazine (Vraylar\textsuperscript{TM}), is an atypical antipsychotic drug commonly used to treat schizophrenia, manic disorder, and bipolar disorder. Cariprazine has a high affinity for D\textsubscript{3}R and D\textsubscript{2}R. However, studies have shown a higher selectivity for the D\textsubscript{3}R, suggesting it to be a viable pharmacotherapeutic for SUDs, including AUD.\textsuperscript{[91,92]}
Drug Dosing

Cariprazine was administered at 0.018, 0.056, 0.18, 0.32, and 0.56 mg/kg, using a Latin square design to control any variability in the experimental procedure. The vehicle used to dissolve this drug was 0.9% NaCl (physiological saline). Cariprazine and the vehicle were administered via intraperitoneal (i.p.) injections. Each injection depends on each mouse's body weight, in which all solutions were given at a 10 ml/kg volume. The Latin square design of Cariprazine used in this study is shown in Table 7.
Table 7

Cariprazine Drug Dosing: Latin Square Design

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 9</th>
<th>Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1A1</td>
<td>Vehicle</td>
<td>0.018 mg/kg</td>
<td>0.056 mg/kg</td>
<td>0.18 mg/kg</td>
<td>0.32 mg/kg</td>
<td>0.56 mg/kg</td>
</tr>
<tr>
<td>C1A2</td>
<td>0.018 mg/kg</td>
<td>0.056 mg/kg</td>
<td>0.18 mg/kg</td>
<td>0.32 mg/kg</td>
<td>0.56 mg/kg</td>
<td>Vehicle</td>
</tr>
<tr>
<td>C1A3</td>
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<td>0.18 mg/kg</td>
<td>0.32 mg/kg</td>
<td>0.56 mg/kg</td>
<td>Vehicle</td>
<td>0.018 mg/kg</td>
</tr>
<tr>
<td>C1A4</td>
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<td>0.32 mg/kg</td>
<td>0.56 mg/kg</td>
<td>Vehicle</td>
<td>0.018 mg/kg</td>
<td>0.056 mg/kg</td>
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<tr>
<td>C2A1</td>
<td>0.32 mg/kg</td>
<td>0.56 mg/kg</td>
<td>Vehicle</td>
<td>0.018 mg/kg</td>
<td>0.056 mg/kg</td>
<td>0.18 mg/kg</td>
</tr>
<tr>
<td>C2A2</td>
<td>0.56 mg/kg</td>
<td>Vehicle</td>
<td>0.018 mg/kg</td>
<td>0.056 mg/kg</td>
<td>0.18 mg/kg</td>
<td>0.32 mg/kg</td>
</tr>
<tr>
<td>C2A3</td>
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<td>0.018 mg/kg</td>
<td>0.056 mg/kg</td>
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</tr>
<tr>
<td>C2A4</td>
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<td>0.18 mg/kg</td>
<td>0.32 mg/kg</td>
<td>0.56 mg/kg</td>
<td>Vehicle</td>
</tr>
</tbody>
</table>
**Animals**

Adult male and female drug-naive C57Bl/6 mice weighing 22-30 grams arrived from Charles River Laboratories. Upon arrival at the Cooper Medical School of Rowan University (CMSRU) vivarium, mice were grouped housed in standard Plexiglass cages in a room maintained on a 12-h light/dark cycle. All mice had continuous access to food and water throughout certain studies and habituated 2 weeks before any experimental procedure. Animals used for the following experiments were cared for per the guidelines of Rowan University's Institutional Animal Care and Use Committee.

**Equipment**

**Operant Chambers**

In these behavioral experiments, operant chambers were used to test the self-administration of palatable food and ethanol. As previously stated, each operant chamber is equipped with a reward receptacle between two nose-poking response holes, an incorrect response, and a correct response. The animals in the food self-administration study received a food reward, a 50/50 mixture of vanilla Ensure and water when their nose poked the left hole. Moreover, no reward was given when the animal's nose poked on the right side. A fixed ratio (FR) system was used to train the mice to nose poke over two months. The animals were trained initially on an FR 1 that escalated to an FR 4 over the course of several weeks prior to any drug testing. Similarly, for the ethanol self-administration studies, the animals were trained to nose poke for a correct response on the left side at a 6% Ethanol and 94% water mixture. The food and ethanol self-administration studies were tested against the drug of interest, cariprazine.
Experimental Procedure

Food Self-Administration Procedure

For the food self-administration studies 8 mice were trained to nose poke for palatable food rewards through operant conditioning. The duration of the training sessions was 120 minutes, 7 days a week, until each animal consistently received rewards at FR 4. Once all the animals were trained at an FR 4, the animals were injected with cariprazine and placed in the operant chambers for testing. Each animal received different daily doses, usually a Latin square design to account for any conditions until they each received one of each dose. The MED-PC program analyzed the disruption of food self-administration caused by cariprazine. They received injections every other day, and on their rest day, they continued their regular training session in food self-administration. After their 120-minute training period, the animals were given free access to food for an hour and fasted overnight for 21 hours until the next training day.

Ethanol Self-Administration Procedure

We repeated the ethanol self-administration studies with the same 8 mice, but the ensure was replaced with water, fading out the ensure incrementally and replacing it with ethanol. They began at a 2% ethanol mixture until they reached a 6% w/v concentration, replacing the ensure with ethanol. Due to the animals not consuming enough ethanol, the procedure for the ethanol self-administration studies were modified. The animals were tested on a FR 2 and extended the training sessions to 240 minutes.

Once they were trained to nose poke under these conditions, we administered cariprazine via i.p. injections before their training session utilizing the same Latin square
design. The purpose of this experiment is to determine the effects of cariprazine on ethanol self-administration.

Data & Results

Food Self-Administration Results

Figure 30

Food Self-Administration Cariprazine Rewards

Note. This graph represents the food self-administration reward values of Cariprazine. The x-axis represents the injected dose of cariprazine (0.018, 0.056, 0.18, 0.32, and 0.56 mg/kg) and the vehicle control. The y-axis is the number of earned rewards. Repeated-measures one-way ANOVA revealed that cariprazine significantly reduced palatable food
self-administration at 0.056, 0.18, 0.32, and 0.56 mg/kg. The 0.018 mg/kg dose had similar rewards to the vehicle. There were no differences between male and female mice. All results are presented as means ± SEM (n=8).

**Figure 31**

*Food Self-Administration Cariprazine Response Rates*

![Graph showing food self-administration response rates of cariprazine.](image)

Note. This graph represents the food self-administration response rate values of cariprazine. The x-axis represents the injected dose of cariprazine (0.018, 0.056, 0.18, 0.32, and 0.56 mg/kg) and the vehicle control. The y-axis represents the rate of responses. Repeated measures of one-way ANOVA revealed that cariprazine significantly decreased response rates at 0.056, 0.18, 0.32, and 0.56 mg/kg. All results are presented as means ± SEM (n=8).
Figure 30 shows the combined results of both male and female mice on the effects of cariprazine on food self-administration studies. The one-way ANOVA analysis shows that cariprazine significantly reduced palatable food reward in all of the doses tested except for the 0.018 mg/kg. Furthermore, no sex differences were observed at any of the doses tested (0.018, 0.056, 0.18, 0.32, and 0.56 mg/kg). Similarly, in Figure 31, the response rates are significantly reduced at 0.056, 0.18, 0.32, and 0.56 mg/kg of cariprazine. These findings suggest that the D₃R and D₂R partial agonist cariprazine may help reduce drug-taking and-seeking behaviors with few disruptive side effects.

Ethanol Self-Administration Results

Figure 32

Ethanol Self-Administration Cariprazine Rewards
Note. This graph represents the ethanol self-administration reward values of cariprazine. The x-axis represents the injected dose of cariprazine (0.018, 0.056, 0.18, 0.32, or 0.56 mg/kg) and the vehicle control. The y-axis is the number of earned rewards. Two-way ANOVA revealed that cariprazine did not significantly reduce ethanol-self administration with tested doses; however, it did reveal a significant sex difference. All results are presented as means ± SEM (n = 8).

Figure 33

Ethanol Self-Administration Cariprazine Response Rates

Note. This graph represents the ethanol self-administration response rate values of cariprazine. The x-axis represents the injected dose of cariprazine (0.018, 0.056, 0.18, 0.32, and 0.56 mg/kg) and the vehicle control. The y-axis is the number of earned
Two-way ANOVA revealed that cariprazine did not significantly alter response rates at any of the doses tested. All results are presented as means ± SEM (n=8).

**Figure 32** represents the analyzed data for the ethanol self-administration studies, revealing cariprazine’s significant sex difference in ethanol administration. The female mice in this study had very low rates of ethanol self-administration, an unexpected problem. From our data, cariprazine was non-significant at any of the doses tested (0.018, 0.056, 0.18, 0.32, and 0.56 mg/kg) in ethanol-seeking models. The two-way ANOVA shows no significant effect for a repeated measure. Similarly, the same results are observed in **Figure 33**, showing no effect on their response rates. The results from this set of experiments are inconclusive because of the surprising sex effect we did not expect. Our results may suggest that cariprazine decreased ethanol-seeking in male mice. However, moving forward, a larger set of animals would need to be tested to evaluate the effects of cariprazine on ethanol-seeking models.

**Discussion**

The D₃R is a rising target of interest for developing new medications to treat SUDs. Alcohol is a primary contributor to the global disease and the leading cause of preventable death. In the United States, AUD is one of the most common psychiatric disorders. The development of new pharmaceutical reagents is a time-consuming, costly, and labor-intensive process. [92] The FDA has approved three medications for treating AUD: naltrexone, acamprosate, and disulfiram. However, the three approved drugs have not effectively targeted the population with AUD. [94] Given the low success rates of current treatment, studying the effects of cariprazine, which has shown to have beneficial
properties, could make it a promising drug for treating AUD. Additionally, cariprazine has yet to be evaluated in animal models with AUD, and other studies have shown promising results for treating other SUDs.

Specifically, this study aimed to determine whether cariprazine alters alcohol-seeking behaviors in animal models with AUD. Our control studies on cariprazine food self-administration revealed that even small doses of cariprazine attenuated their self-administration. These control studies helped separate alcohol-seeking effects from behavioral and appetitive effects. The cariprazine ethanol self-administration studies revealed that the female mice could not self-administer ethanol, which was an unexpected finding. Our male mice results could potentially suggest that cariprazine could reduce ethanol use; nevertheless, our data remains inconclusive due sex differences found. Further experiments would be necessary to fully conclude that cariprazine, possibly through D₃R effects, may have anti-addiction potential.

Studies have shown biological differences between males and females and their influence to respond to alcohol. It has been observed that in both humans and rodents, females escalate alcohol use and develop addictive behaviors more quickly compared to males. The mechanism behind these biological differences remains unclear, which is why our results were surprising. Our results do not support this observation, a reason could be the strain of the animal we used in addition to our protocol strategy. After some analysis for future studies we plan to monitor their progress and perhaps test the animals at different times. The different sexes could prefer different percentages of alcohol, and 6% could have been too high for the female mice. Future studies could also include testing different strains and comparing sexes amongst different mice strains.
Additionally, there is literature that has given the animal access to alcohol instead of water in their housing units, having them more exposed to ethanol throughout the study. Moreover, it would be interesting to see how rats would compare in this study, as they learn much faster and are better candidates for self-administration studies.

In summary, substance abuse continues to escalate in the United States, increasing the need to identify new pharmacotherapeutic treatments to improve the quality of life. The advances in these research avenues are bringing us closer to discoveries that will help target better treatment options for patients.
References


103


